UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Desarrollo y aplicación de técnicas biotecnológicas para el saneamiento, multiplicación, caracterización y conservación de germoplasma de vid (*Vitis vinifera* L.)

TESIS DOCTORAL

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Para Mateo

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Esta tesis se ha realizado en el marco de los proyectos de investigación RTA-2011-00067 (INIA) y CGL2015-70843-R (MINECO) cofinanciados con fondos FEDER.

Resumen

La vid es un cultivo de propagación vegetativa y de gran importancia económica cuyo cultivo se distribuye mundialmente por zonas templadas. Su producción va destinada principalmente a la elaboración de vino, seguido de la producción de uva de mesa. Aunque existen miles de variedades, una decena de ellas son las que se utilizan mayoritariamente a nivel mundial lo que conlleva a una pérdida de variabilidad. Desde la llegada de la filoxera, el cultivo de la vid se realiza mediante injerto en pies resistentes a este áfido. Según la Comisión Directiva 2005/43/EC por la que se modifican los anexos del Consejo Directivo 68/193/EEC, el material de propagación de vid debe estar libre de los siguientes virus: virus del entrenudo corto (GFLV), virus del enrollado 1 (GLRaV-1), virus del enrollado 3 (GLRaV-3), virus del jaspeado (GFkV) y virus del mosaico del arabis (ArMV). En este trabajo se han evaluado y desarrollado distintas metodologías relacionadas con el cultivo *in vitro* de plantas encaminadas al saneamiento, multiplicación y conservación de germoplasma de vid. La conservación de variedades de vid *in vitro* es muy importante para complementar la conservación de germoplasma de las colecciones de campo. Para ello es importante seleccionar el material a conservar, determinar su estado sanitario, sanear si es el caso y establecer las condiciones adecuadas para su conservación. En un primer capítulo de esta tesis (Capítulo 1) se describen los ensayos realizados para inducir la embriogénesis somática en 16 variedades de vid que incluyen seis variedades infectadas con los virus GFLV, GLRaV-3 y GFkV. La embriogénesis somática ha sido descrita en vid como una metodología útil para el saneamiento de plantas infectadas con virus y es la vía común de regeneración adventicia. Protocolos eficientes de regeneración son necesarios para abordar su mejora mediante transformación genética. En este trabajo se utiliza como material de partida semillas cortadas y se han obtenido plantas libres de virus y porcentajes de regeneración elevados en todas las variedades evaluadas. La utilización de marcadores microsatélites nos ha servido para seleccionar entre las plantas regeneradas aquellas que muestran el mismo genotipo que las plantas de partida. Por otra parte, en este trabajo se ha desarrollado un protocolo de micropropagación a partir de planta sana de la variedad 'Monastrell' cultivada *in vitro* obteniéndose tasas de multiplicación elevadas y plantas que se han aclimatado a condiciones de campo (Capítulo 2). El medio de cultivo MW, utilizado para el desarrollo de las plantas, se ha evaluado en otras 16 variedades y también se ha comparado el crecimiento en este medio y en

variaciones de éste, como son la disminución de la concentración de azúcar a la mitad y la eliminación del ácido indolbutírico. La finalidad de estos ensayos ha sido determinar para cada variedad qué condiciones son las más adecuadas para mantener el germoplasma vegetal en cultivo *in vitro* activo, pero alargando el tiempo entre subcultivos optimizando costes (Capítulo 3). En el Capítulo 4 se incluye un trabajo de divulgación en el que se resumen los pasos que se están siguiendo en el marco del proyecto MINECO CGL2015-70843-R para localizar, identificar, descartar o detectar la presencia de los virus anteriormente comentados, sanear si es el caso, y conservar *in vitro* las variedades de interés en las que se lleva a cabo una identificación utilizando marcadores SSR y un análisis de variabilidad genética cuando se dispone de varias accesiones. A modo de ejemplo, en este trabajo se publica el perfil de marcadores microsatélites para dos de las variedades colectadas y/o conservadas, la variedad 'Esclafacherre' ('Esclafagerres') de la que se han localizado únicamente dos cepas en dos zonas distintas de la Comunidad Valenciana, y accesiones de 'Valencí Blanc' en las que se ha detectado diferencias para el marcador VVMD32 que agrupa las accesiones de Alicante por una parte, y al resto de accesiones por otra. También se ha validado un protocolo de crioconservación en la variedad 'Esclafacherre'. Por último, el Capítulo 5 hace referencia a un estudio llevado a cabo para determinar el estado actual de la aplicación de la transformación genética en vid, centrándonos en los factores limitantes de las metodologías empleadas. La transformación genética es una herramienta de gran potencial para la mejora de los cultivos, principalmente en plantas de propagación vegetativa.

Esta tesis doctoral se ha realizado en el marco de la Unidad Asociada que mantienen los directores de la tesis, especialistas en virología y cultivo *in vitro*, en el marco del proyecto RTA-2011-00067 coordinado por el Dr. Olmos y el proyecto MINECO CGL2015-70843-R que dirige la Dra. Gisbert, ambos cofinanciados con fondos FEDER.

Abstract

Grapevine is a crop of vegetative propagation with great economical importance which is distributed worldwide in temperate zones. It is mainly used for wine and table grape production. Despite the fact thousands of varieties exist only few are commonly used, producing a loss of variability. Since the arrival of the phylloxera aphid the grapevine culture is performed using resistant rootstocks. The Directive Commission 2005/43/EC amending the Directive Council annexes 68/193/EEC, indicates that grapevine material should be free of these viruses: Grapevine fanleaf virus (GFLV), Grapevine leafroll associated virus 1 (GLRaV-1), Grapevine leafroll associated virus 3 (GLRaV-3), Grapevine fleck virus (GFkV) and Arabis mosaic virus (ArMV). In this work different methodologies related with *in vitro* culture were evaluated and developed with the aim of sanitate, mutiplicate and preserve grapevine germplam. *In vitro* preservation of grapevine varieties is a complementary stratregy of field preservation. In a preservation program it is important the selection of the starting material, determine its sanitary status, sanitate if it is necessary and, establish the appropriate *in vitro* conditions for its preservation. In the first chapter of this thesis (Chapter 1) the assays carried out for inducing somatic embryogenesis in 16 grapevine varieties which include six that were infected by the viruses GFLV, GLRaV-3 and GFkV are described. In grapevine, somatic embryogenesis has been described as a usefull methodology for virus sanitation and is the common pathway to adventitious regeneration. Efficient regeneration prototocols are necessary to address breeding by genetic transformation. In this work, cut seed are used as starting material and virus-free plants and high precentages of regeneration were obtained in all the varieties evaluated. The use of microsatellite markers has been useful to select those plants with the same genotype than the mother plant. From the other side, in this work, a micropropagation protocol starting from a healthy plant of the cultivar 'Monastrell' was developed and high multiplication rates with good adaptation to field conditions were obtained (Chapter 2). The culture medium MW was evaluated in 16 grapevine varieties as well as the following modifications: reduction in a half of the sugar content and elimination of the Indolebutyric Acid. The aim of these assays was to determine for each variety which conditions are the most appropiate to maintain in active *in vitro* culture this germplasm, lengthen the time between subcultures for reducing costs (Chapter 3). In Chapter 4 it is included a divulgation report that summarized the steps followed in the MINECO proyect CGL2015-70843-

R for localizing and identifying grapevine varieties; discard or detect the presence of the viruses, sanitizing if it is necessary and, conserve *in vitro* the most interesting accessions. Microsatellite markers were used for genotyping. Analysis of variability were performed when several accessions were available. As an example, in this work it is published the microsatellite profile for two accessions of the endangered cultivar 'Esclafacherre' ('Esclafagerres') localized in two fields of the Valencian Community, and those of several accessions of 'Valencí Blanc' which differed for the VVMD32 marker, grouping the Alicante accessions and the rest of accessions from other origins. It was also validated a cryoconservation protocol for the 'Esclafacherre' variety. By last, Chapter 5 makes reference to a review performed with the goal to know the state of the art of genetic transformation in grapevine focusing on the main limiting factors of the employed methodologies. Genetic transformation is a potential tool for crop breeding, mainly in vegetative propagation plants.

This thesis has been carried out within the framework of an Associated Unity shared by the thesis directors Dr. Gisbert and Dr. Olmos, specialized in *in vitro* culture and virology, respectively. The firts lead the MINECO project CGL2015-70843-R, the second coordinated the project RTA-2011-00067, both co-financed by FEDER funds.

Resum

La vinya és un cultiu de propagació vegetativa i de gran importància econòmica que es distribueix mundialment per zones temperades. La seua producció va destinada principalment a l'elaboració de vi, seguit de la producció de raïm de taula. Encara que existeixen milers de varietats, una desena d'elles són les que s'utilitzen majoritàriament a nivell mundial, el que comporta una pèrdua de variabilitat. Des de l'arribada de la fil·loxera, el cultiu de vinya es realitza mitjançant l'ampelt en peus resistents a aquest àfid. Segons la Comissió Directiva 2005/43/EC per la qual es modifiquen els anexos del Consell Directiu 68/193/EEC, el material de vinya ha d'estar lliure dels següents virus: virus de l'entrenús curt (GFLV), virus de l'enrotllat 1 (GLRaV-1), virus de l'enrotllat 3 (GLRaV-3), virus de jaspejat (GFkV) i virus del mosaic de l'arabis (ArMV). En este treball s'han avaluat i desenvolupat diferents metodologies relacionades amb el cultiu *in vitro* de plantes encaminades al sanejament, multiplicació i conservació de germoplasma de raïm. La conservació de varietats de raïm *in vitro* és molt important per a complementar la conservació de germoplasma en les col·leccions de camp. Per això és important seleccionar el material a conservar, determinar el seu estat sanitari, sanejar si cal i establir les condicions adequades per a la seua conservació. En el primer capítol d'esta tesi (Capítol 1) es descriuen els assaigs realitzats per a induïr l'embriogènesi somàtica en 16 varietats de raïm que inclouen sis varietats infectades amb els virus GFLV, GLRaV-3 i GFkV. En vinya, l'embriogènesi somàtica ha sigut descrita com una metodologia útil per al sanejament de plantes infectades amb virus i és la via comú de regeneració adventícia. Protocols eficients de regeneració són necessaris per a abordar la seua millora mitjançant transformació genètica. En este treball s'utilitza com a material de partida llavors tallades i s'han obtingut plantes lliures de virus i percentatges de regeneració elevats en totes les varietats avaluades. La utilització de marcadors microsatèl·lits ens ha servit per a seleccionar entre les plantes regenerades aquelles que mostren el mateix genotip que les plantes de partida. Per altra banda, en este treball s'ha desenvolupat un protocol de micropropagació a partir de planta sana de la varietat 'Monastrell' cultivada *in vitro*, obtenint-se taxes de multiplicació elevades i plantes que s'han aclimatat a condicions de camp (Capítol 2). En el medi de cultiu MW, utilitzat per al desenvolupament de les plantes, s'han avaluat un total de 16 varietats i també s'ha comparat el creixement en este medi i en variacions d'este, com son la disminució de la concentració de sucre a la meitat i la eliminació de l'àcid

indolbutíric. La finalitat d'estos assaigs ha sigut determinar per a cada varietat quines condicions són les més adequades per a mantindre el germoplasma vegetal en cultiu *in vitro* actiu però allargant el temps entre subcultius i optimitzant costos (Capítol 3). En el Capítol 4 s'inclou un treball de divulgació en el que es resumeixen els passos que s'estan seguint en el marc del projecte MINECO CGL2015-70843-R per a localitzar, identificar, descartar o detectar la presència dels virus anteriorment comentats, sanejar si cal, i conservar *in vitro* les varietats d'interés en les que es porta a cap una identificació utilitzant marcadors SSR i l'anàlisi de la variabilitat genètica quan es disposa de diverses accessions. A mode d'exemple, en estre treball es publica el perfil de marcadors microsatèl·lits per a dos de les accessions col·lectades de la varietat en perill d'extinció 'Esclafacherre' ('Esclafagerres') de la que s'han localitzat únicament dos ceps en dues zones diferents de la Comunitat Valenciana. També, es mostra el genotipat d'accessions de 'Valencí Blanc' en les que s'han detectat diferències per al marcador VVMD32 que agrupa les accessions d'Alacant per una banda, i a la resta d'accessions per altra. També s'ha validat un protocol de crioconservació en la varietat 'Escalfacherre'. Per últim, el Capítol 5 fa referència a un estudi portat a terme per a determinar l'estat actual de l'aplicació de la transformació genética en raïm, centrant-nos en els factors limitants de les metodologies empleades. La transformació genética és una ferramenta de gran potencial per a la millora dels cultius, principalment en plantes de propagació vegetativa.

Esta tesi doctoral s'ha realizat en el marc de la Unitat Associada que mantenen els directors de la tesi, especialistes en virologia i en cultiu *in vitro*, en el marc del projecte RTA-2011-00067 coordinat pel Dr. Olmos i en el projecte MINECO CGL2015-70843-R que dirigeix la Dra. Gisbert, ambdós cofinanciats amb fons FEDER.

Índice

Palabras clave: vid, saneamiento, virus, embriogénesis somática, multiplicación, micropropagación, medio de cultivo, caracterización, marcadores moleculares, conservación, germoplasma.

Abreviaturas

ArMV: Arabis mosaic virus/ virus del mosaico del arabis

BAP: Bencilaminopurina

D.O.: Denominación de Origen

D.O.Ca.: Denominación de Origen Calificada

D.O.P.: Denominación de Origen Protegido

GFkV: Grapevine fleck virus/ virus del jaspeado

GFLV: Grapevine fanleaf virus/ virus del entrenudo corto

GLRaV-1: Grapevine leafroll associated virus 1/ virus del enrollado 1

GLRaV-3: Grapevine leafroll associated virus 3/ virus del enrollado 3

GVA: Grapevine vitivirus A

ha: Hectárea

IBA: Ácido indulbutírico

IMIDRA: Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario

OIV: Organización Internacional del Vino

PCR: Polymerase Chain Reaction

PVS2: Plant Vitrification Solution 2

RFLP: Restriction Fragment Lenght Polymorphism

SNP: Single Nucleotid Polymorphism

SSR: Marcadores microsatélite

TDZ: Tidiazurón

1. Introducción

1.1 Biología y clasificación de la vid

La vid cultivada (*Vitis vinifera* L.) pertenece al género *Vitis* cuyas especies se distribuyen desde climas fríos hasta cálidos. Las principales áreas de este cultivo se sitúan entre los paralelos 30º y 50º de latitud norte, y 30º y 40º de latitud sur. A su vez este género está incluido en la gran familia *Vitaceae*. Dentro del género *Vitis* se distinguen dos subgéneros (VIVC 2017), ambos diploides diferenciados por su dotación cromosómica; *Muscadinia*, con una dotación cromosómica 2n=40, y *Euvitis*, con una dotación cromosómica 2n=38 (Fig. 1).

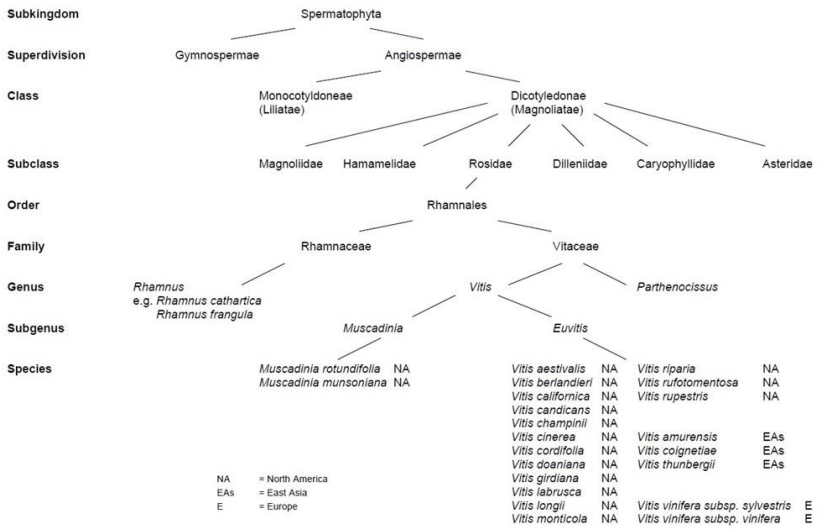

Fig. 1. Árbol taxonómico del género *Vitis*. En este árbol se incluyen las especies más comunes de los subgéneros *Muscadinia* y *Euvitis* (VIVC 2017)

El subgénero *Muscadinia* incluye 3 especies: *Muscadinia rotundifolia*, *Muscadinia munsoniana* y *Muscadinia popenoeii*, presentes en zonas cálidas y templadas del sudeste de América del Norte: Estados Unidos y México. Solo *M. rotundifolia* se cultiva en Europa por ser resistente a enfermedades criptogámicas, ya que presenta

interés en programas de mejora. El subgénero *Euvitis* incluye a casi todas las vides cultivadas. Dentro de éste se distinguen tres grupos: el americano, el asiático y el euroasiático.

Grupo americano: lo forman una veintena de especies muy resistentes a filoxera, por lo que generalmente se utilizan como portainjertos o para obtener híbridos productores directos (HPD) (Alleweldt & Possingham 1988). Las especies *Vitis riparia*, *Vitis rupestris*, los híbridos interespecíficos de éstas, y *Vitis berlandieri* han sido utilizadas como portainjertos resistentes a la filoxera (Pouget 1990). *Vitis champini* también ha sido utilizada como portainjerto por su adaptabilidad a suelos salinos (Lowe & Walker 2006), así como *Vitis cinerea* por su resistencia a la infección vírica a través de nematodos (Schmid et al. 2003). *Vitis labrusca* es la única especie de este grupo con aptitudes viníferas.

Grupo asiático: formado por algo más de 20 especies. No son resistentes a filoxera y tienen poca capacidad vinícola. *Vitis amurensis* es la más común, además es resistente al mildiu y al frío.

Grupo euroasiático: con solo una especie, *V. vinifera* L. que incluye todas las variedades de vid cultivadas en el mundo, tanto de mesa como de vinificación. Se cultiva en zonas templadas, se multiplica bien vegetativamente y es sensible a filoxera y a enfermedades criptogámicas (Reynier 2002), por lo que se hace necesario normalmente la utilización de portainjertos para su cultivo. Existen dos formas de *V. vinifera*, la *sylvestris* y la *sativa*, que algunos consideran como dos especies y otros como dos subespecies (This et al. 2006). Si nos basamos en su morfología las consideraríamos dos especies distintas (*V. sylvestris*, sp. Hegi Gmelin y *V. vinifera*, sp. L.), pero si vemos estas diferencias como el resultado de un proceso de domesticación las consideraríamos dos subespecies dentro de una misma especie (*V. vinifera* L. subsp. *sylvestris* y subsp. *sativa*) (De Andrés et al. 2012).

1.2 Origen, domesticación e importancia económica

El origen de la vid no se conoce con exactitud, pero tanto en América como en Europa se han encontrado fósiles del periodo Eoceno de los géneros *Ampelopsis* y *Cissus*, pertenecientes a la familia *Vitaceae* y de diferentes especies del género *Vitis*. También

se han localizado restos de polen y fósiles de plantas de vid datados del Mioceno en otros países como Alemania, Inglaterra, Islandia, Francia, América del Norte, Alaska y Japón (Turner 1968, Van der Burgh 1974, Galet 2000, Vanhoorne 2005). En Fairon-Demaret & Smith (2002) también se describe la localización en Bélgica de dos tipos de semillas de vid: con chalaza lisa (*Vitis rectisulcata*) y semillas con chalaza rugosa (*Vitis longisulcata*). Las primeras serían similares a las semillas de vid de hoy en día y las rugosas tendrían más parecido a las que presentan las especies del subgénero *Muscadinia*. La aparición de estos fósiles indica que el género *Vitis* ya se encontraba ampliamente distribuido por el hemisferio norte a finales del Terciario y que la separación de *Euvitis* y *Muscadinia* pudo ocurrir al inicio de este periodo. Se cree que las especies del subgénero *Muscadinia* se extinguieron en Europa durante las glaciaciones del Cuaternario, por eso este subgénero se distribuyó ampliamente durante el Terciario y no en el Cuaternario, como sí lo hizo el subgénero *Euvitis*.

La Teoría de la Deriva Continental propone que, en el Cuaternario, Norteamérica se separó de Eurasia y dividió el subgénero *Euvitis* en dos grupos de especies, las euroasiáticas y las americanas. Esto explica por qué se observan semejanzas entre algunas especies americanas y asiáticas, como *V. labrusca* que se parece mucho a *Vitis coignetiae*, o *Vitis caribeae* que tiene una alta semejanza con *Vitis lanata* (Levadoux et al. 1962). Esto sugiere que estas especies evolucionaron de un ancestro común, pero en diferentes continentes. Muchas de las especies del género *Vitis* surgieron durante las glaciaciones del Cuaternario. De Lattin (1939), sugiere que la distribución de *Vitis* es compatible con la fragmentación de poblaciones por los glaciares, pues las vides solo podrían sobrevivir en zonas protegidas de la influencia glaciar, aisladas, para que se dieran las condiciones más favorables para la especiación. Tras la retirada del hielo después de cada glaciación, se recolonizaron los espacios ocupados por los glaciares por lo que las vides que habrían evolucionado por separado se volvieron a encontrar. Esto explicaría el gran número de especies dentro del género *Vitis* (Galet 1988).

1.2.1 Evolución de las variedades de vid cultivadas

Existen miles de cultivares de *V. vinifera* (Robinson et al. 2012), pero son las variedades económicamente importantes como ‘Cabernet Sauvignon’, ‘Merlot’, ‘Airén’, ‘Tempranillo’, ‘Chardonnay’, ‘Syrah’, ‘Garnacha Tinta’, ‘Sauvignon Blanc’,

'Trebbiano Toscano' y 'Pinot Noir' las que dominan el mercado mundial (Anderson & Aryal 2013). En cuanto a la forma silvestre es rara de encontrar en su hábitat natural y se extiende desde Portugal a Turkmenistan, y desde las orillas del Rin hasta los bosques del norte de Túnez (Levadoux 1956, Arnold et al. 1998, McGovern 2003).

La domesticación de la vid parece que fue ligada al descubrimiento del vino (Royer 1988, McGovern 2003) pero no está claro qué proceso se produjo primero. Probablemente, se produjo vino de forma accidental a causa del almacenamiento de uva durante los meses de invierno. La vid ha sido uno de los primeros cultivos en domesticar, haciendo que *V. vinifera sylvestris* evolucionara y se convirtiera en la vid cultivada de hoy en día, *V. vinifera sativa*. Se considera "planta domesticada" aquella cuyo proceso reproductivo está controlado por el hombre en su propio beneficio. En el proceso de domesticación, la vid sufrió diversos cambios que condujeron a un mayor contenido de azúcares para una mejor fermentación, un rendimiento mejorado y una producción más regular. En este proceso se realizó selección a favor del tamaño del grano y del racimo, y el paso de plantas dioicas a monoicas hermafroditas fue crucial, pues así se garantizaba la polinización y reproducción (Levadoux 1956, Terral 2002). También hubo cambios en la forma y tamaño de las semillas, pero su significancia biológica es desconocida (Marinval 1997). No se sabe si estos cambios se produjeron en un periodo largo de tiempo (cruzamientos sexuales, selección natural o humana) o si fueron relativamente rápidos (mutaciones, selección y propagación por vía vegetativa). Lo más probable es que fuera una combinación de ambos procesos (This et al. 2006) y, por tanto, se pueden contemplar dos hipótesis que explican el origen de la vid cultivada:

1. La primera hipótesis atribuye un único origen de domesticación a partir de las poblaciones de vid silvestre, cuyos cultivares seleccionados se extenderían a otras regiones (Olmo 1976).

2. La segunda hipótesis plantea varios orígenes de domesticación en los cuales participan un gran número de variedades silvestres en diferentes zonas geográficas y en un tiempo más prolongado (Grassi et al. 2003, Arroyo-García et al. 2006, Imazio et al. 2006).

Las evidencias más antiguas de producción de vino las proporcionó el descubrimiento de vasijas de arcilla en Irán (en el norte de la cadena montañosa Zagros), datadas del 7000-6000 a.C y en las que se detectó grandes cantidades de ácido tartárico (McGovern & Rudolph 1996). A causa del comercio y las migraciones, el cultivo de la vid se fue extendiendo hacia el oeste del Mediterráneo. También se han encontrado semillas de vid domesticada en Georgia y Turquía de hace unos 8000 años y restos de semillas del Neolítico en el oeste de Europa y en Francia (Marinval 1997). En Oriente Próximo, los restos arqueológicos más antiguos se han localizado en una región conocida como Transcaucásica, donde hay una gran variedad genética de vides silvestres (Bouquet 2011). En Europa también se registraron restos paleobotánicos de vid silvestre, los encontrados en España datan del 3000 a.C. (Núñez & Walker 1989).

1.2.2 Expansión del cultivo de la vid

Desde los lugares en donde se domesticó este cultivo hubo una expansión gradual hacia regiones adyacentes como Egipto o la Baja Mesopotamia (5500-5000 a.C.) que tuvieron una gran actividad con este cultivo, y se inició el comercio de vino llegando a Grecia. Posteriormente, los griegos introdujeron el vino por el sur de Italia, aunque allí ya había vides cultivadas por los etruscos (Amouretti 1992). Los romanos fueron los primeros en dar nombres a las diferentes variedades de vid (Roxas 1814) y con su imperio las dispersaron por las regiones templadas de Europa llegando incluso al norte de Alemania (Johnson 2005, This et al. 2006). El cultivo de vid también llegó a Japón hace 3200 años y en el siglo II llegó a China (Royer 1988).

Al final del Imperio Romano, los cultivos de vid se encontraban prácticamente en los mismos lugares que se encuentran hoy en día en Europa (This et al. 2006). Durante la Edad Media fue la Iglesia Católica la encargada de extender el cultivo al norte de Europa y de intercambiar germoplasma a través de las cruzadas. La extensión del Islam al norte de África, España y el este del Mediterráneo también tuvo un papel importante en la dispersión de la vid, sobretodo, de uva de mesa (Royer 1988). Después del Renacimiento (siglo XVI) la vid colonizó los países del Nuevo Mundo. Los misioneros la introdujeron en América, primero como semillas y posteriormente mediante esquejes que también se introdujeron en el sur de África, Australia y Nueva Zelanda, llegando al norte de África en el siglo XIX. Tras la expansión de este cultivo, a final del siglo XIX, la filoxera entró en Europa desde América y provocó

una gran catástrofe en el viñedo europeo, sensible a este áfido, reduciendo drásticamente la diversidad de variedades (This et al. 2004). Desde entonces el cultivo de la vid tuvo que realizarse utilizando portainjertos resistentes. Este hecho supuso una gran pérdida de diversidad. En los últimos 50 años la diversidad en los cultivos de vid ha ido disminuyendo por la globalización de las compañías viníferas que solo trabajan con algunas variedades económicamente viables, dejando a un lado variedades locales minoritarias, y la aparición de las Denominaciones de Origen Protegido (D.O.P.) que permiten el uso de un conjunto limitado de variedades. No obstante, hoy en día se están intentando recuperar variedades locales por el interés que puedan tener en mejora varietal (Santana et al. 2008).

1.2.3 Distribución del cultivo a nivel mundial y en España

La vid es un cultivo exigente y condicionado por el clima. Es sensible a las heladas de invierno y de primavera, pues afectan tanto a su desarrollo vegetativo como a la maduración de sus frutos, que necesitan condiciones de iluminación y temperaturas elevadas. Por eso, como hemos mencionado anteriormente, las principales áreas de este cultivo se sitúan entre los paralelos 30º y 50º de latitud norte, y 30º y 40º de latitud sur. Fuera de este rango se pueden dar variaciones climáticas que permiten su cultivo, como en algunas zonas con proximidad a grandes masas de aguas o accidentes orográficos que afectan a la distribución de los viñedos. Más de la mitad del cultivo de vid mundial se sitúa en Europa, aunque hoy en día la sobreproducción ha provocado el arranque de muchos viñedos. Es el caso de lo ocurrido el año 2010, cuando la Unión Europea tuvo que adaptarse a las disposiciones de la Organización Común del Mercado Vitivinícola (OCM).

El resto de continentes está aumentando o manteniendo la superficie del cultivo (Fig. 2), especialmente en Asia, donde se produce una gran cantidad de uva para productos no vinificados. Irán, Turquía y Siria son los países dentro del continente asiático que más han aumentado la superficie de viñedo, junto a China. En África los países que más superficie tienen de este cultivo son Egipto y Sudáfrica. En Sudamérica, Argentina y Brasil son los países con más superficie de cultivo, aunque disminuye lentamente año a año mientras que Chile sigue manteniendo la misma superficie de viñedo desde 2014. La disminución de la superficie de vid cultivada en Europa contrasta con el aumento en países sudamericanos, Estados Unidos o Nueva Zelanda,

que han iniciado hace relativamente poco el cultivo de vid de forma pujante (OIV 2016).

Desde el punto de vista económico, la vid es el cultivo más importante, con más de 7.2 millones de hectáreas (ha) cultivadas en todo el mundo que producen unos 74.5 millones de toneladas de uva (Castelluci 2011, FAOSTAT 2014). La mayor parte de esta producción se destina a la elaboración de vino. Los principales países productores son China, Estados Unidos e Italia (12.5, 7.15 y 6.9 millones de toneladas, respectivamente) (FAOSTAT 2014).

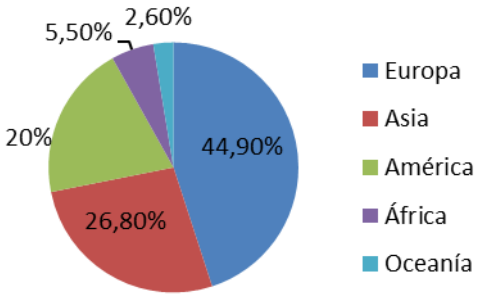

Fig. 2. Proporción del área dedicada al cultivo de vid en cada continente (FAOSTAT 2014)

España ha sido, desde hace siglos, uno de los países vitivinicultores más importantes junto a Francia o Italia y ha contado con una gran diversidad de cultivares de vid. Existen descripciones de variedades españolas desde el periodo romano. Así, Catón (234-149 a.C.) describe en su libro "*De re rustica*" ocho variedades, Virgilio (79-10 a.C.) describe en *"Las Geórgicas"* 7 más y Columela (siglo I d.C.) cita en su libro "*De re rustica*" otras 58 variedades de vid; después, en el libro "*Naturalis Historia*" de Plinio II (23-79 d.C.) se describen 83 variedades según su morfología. Posteriormente, en 1513, Alonso de Herrera en su libro "*Agricultura General*" cita 14 linajes de vid según el color de la baya y De Rojas Clemente, en 1832, describió 119 variedades de vid cultivadas en España. Tras la crisis filoxérica, en 1942, Comenge publicó un resumen de las variedades de vid españolas teniendo en cuenta la pilosidad

de las hojas y en 1954 Marcilla describe las variedades cultivadas en cada región del país en su "Tratado práctico de viticultura y enología española". Según Duque (1992), en 1971, Hidalgo y Candela describen 312 variedades en el artículo "Contribución al conocimiento del inventario vitícola Nacional".

Actualmente España cuenta con 70 Denominaciones de Origen (D.O.), también llamadas D.O.P. Este sistema se utiliza para reconocer una calidad diferenciada en los vinos, con características propias y diferenciales según la zona en la que se producen las materias primas y se elaboran los productos. También existe una categoría superior, la Denominación de Origen Calificada (D.O.Ca.), que además de cumplir los requisitos necesarios de las D.O., deben de presentar otros, como tener un control desde la producción hasta la comercialización del vino, que se tiene que vender embotellado, y tiene que existir una delimitación cartográfica, por municipios, de los terrenos aptos para elaboración de los vinos con derecho a D.O.Ca. En España contamos con dos D.O.Ca., la de la Rioja que consiguió su mención calificada en 1991, y la del Priorat, situada en la provincia de Tarragona, que consiguió su mención en el año 2009. A pesar de la importancia del sector la superficie de cultivo ha ido disminuyendo. En el año 1980, España contaba con una superficie de viñedo de 1.642.622 ha (OEMV 2017), casi 700.000 ha más que las existentes en 2016 (955.717 ha). Castilla la Mancha es la comunidad autónoma con mayor superficie cultivada (473.331 ha) que representa el 49.5% de total. Le siguen de lejos Extremadura (83.039 ha), Castilla y León (64.473 ha) y la Comunidad Valenciana (61.367 ha), que normalmente ha ocupado el tercer puesto, Cataluña (55.118 ha) y La Rioja (52.076 ha). Los datos muestran que del total de superficie de viñedo en España, el 62.8% plantado corresponde a cultivo de secano, mientras que el 37.2% restante lo es de regadío. El cultivo de secano se ha mantenido estable mientras que el de regadío ha crecido un 8.4%. La superficie solo ha crecido en La Rioja (66.6%) y en el País Vasco (56.5%). La caída global la lidera Castilla la Mancha, con una reducción del 37.5%. También desciende la superficie del cultivo en Andalucía, La Comunidad Valenciana, Aragón, Castilla y León y Cataluña (Fig. 3).

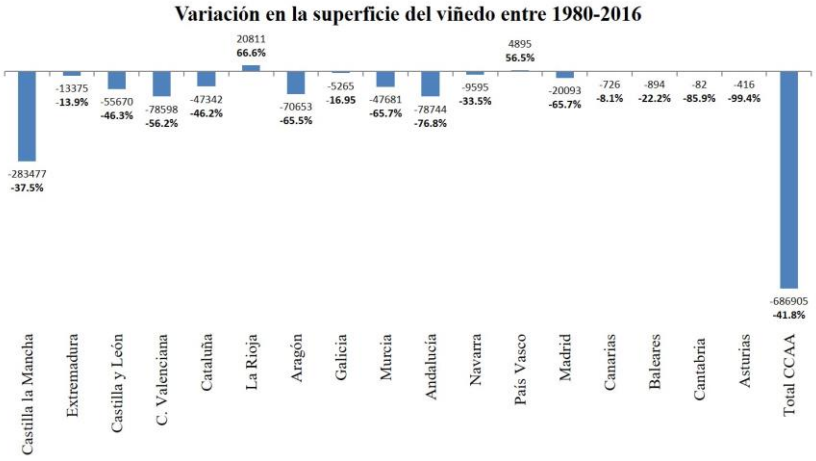

Fig. 3. Variación en la superficie del viñedo entre 1980-2016 (OEMV 2017)

La Comunidad Valenciana ha sido una zona vitícola importante desde tiempos históricos con una gran diversidad de variedades de vid en las provincias de Alicante y Valencia (Sanz-Bremón 1882). En 1889 se citan en la provincia de Valencia más de 150 variedades que incluyen variedades tintas, blancas y rosadas, siendo, en comparación con otras provincias, una de las más ricas (DGAIC 1889). En este momento existen tres D.O. para la producción de vino: 1) D.O. 'Alicante' (http://www.vinosalicantedop.org/), que se divide en dos subzonas; 'La Marina' al norte de la costa alicantina y 'El Vinalopó' con D.O. para uva de mesa (D.O. 'Uva de mesa del Vinalopó', http://www.uva-vinalopo.org/wp/) que se extiende hasta los límites con Castilla la Mancha y Murcia; 2) la D.O. 'Valencia' (http://www.dovalencia.info/) que consta de cuatro subzonas, 'Valentino' situada al noroeste de la provincia, con una altitud máxima de 550 metros sobre el nivel del mar y con la mayor extensión, 'Alto Turia' situada al oeste de 'Valentino' y con una altitud máxima de 625 metros, 'Moscatel' que parte de límites occidentales de la ciudad hacia el interior y que alcanza una altitud de 100 metros, y 'Clariano' aislada de las otras zonas de cultivo y que limita al norte con Valencia capital y al sur con Játiva y Gandía; y 3) la D.O. 'Utiel-Requena' (http://www.utielrequena.org/) que consta de nueve municipios; Camporrobles, Caudete de las Fuentes, Fuenterrobles, Siete Aguas, Sinarcas, Utiel, Requena, Venta del Moro, y Villargordo del Cabriel; uno de ellos (Requena) incluído en la D.O. Cava. Por último, en Castellón se dispone de una Indicación Geográfica Protegida (http://www.igpcastello.com/).

1.3 Identificación y caracterización de variedades de vid

La correcta identificación y caracterización de las variedades de vid es esencial cuando se plantan viñedos, en la producción de vino, para el manejo de las colecciones de germoplasma, en la elección de parentales para cruces controlados y para la protección legal de nuevas variedades (Thomas et al. 1994). En este cultivo es habitual que se den casos de sinonimias (se atribuyen diferentes nombres a una misma variedad en diferentes áreas de cultivo) y de homonimias (se atribuye un mismo nombre a diferentes variedades). Con estos fines se han utilizado distintas metodologías que han ido evolucionando con el tiempo para así evitar una incorrecta identificación de cultivares.

1.3.1 Ampelografía

Se basa en la observación de determinados rasgos del cultivar, y junto a la ampelometría, que se basa en la medida precisa de características fenotípicas de la vid, permiten la identificación de diferentes variedades. Tradicionalmente, los cultivares de vid se han caracterizado e identificado mediante esta técnica, que contiene descriptores estándares. Hoy en día hay 150 descriptores incluidos para la caracterización de variedades. La Organización Internacional del Vino (OIV), la Union International pour la Protectiones des Obtentions Végétales (UPOV) y la International Board for Plant Genetic Resources (IBPGR) se pusieron de acuerdo en establecer una metodología común para la descripción ampelográfica de variedades, que sirve para identificarlos, caracterizarlos, evaluarlos, proteger los derechos de autor y para un correcto etiquetado de las entradas en los bancos de germoplasma. Se aprobó una lista de 130 caracteres morfológicos para su utilización internacional, aunque hay 14 descriptores prioritarios para una rápida caracterización varietal. Una correcta descripción ampelográfica tiene en cuenta el estado de desarrollo de la planta, el estado de salud y las condiciones ambientales (Cipriani et al. 1994), es por eso que puede dar lugar a errores, pues la expresión de caracteres morfológicos depende de todo lo anteriormente citado.

1.3.2 Métodos bioquímicos

Precedieron al uso de marcadores moleculares, fueron muy usados en los años 90. Se desarrollaron como un método complementario a la caracterización ampelográfica, incluyendo análisis de isoenzimas, componentes aromáticos y fenoles, así como análisis serológico de proteínas del polen (Tomiç et al. 2013). Este tipo de análisis se utilizó mucho para ver diferencias entre cultivares, pero la expresión de las enzimas depende del momento del ciclo biológico de la planta y del tejido utilizado para el análisis, al igual que en el caso de la ampelografía (Subden et al. 1987).

1.3.3 Métodos moleculares

Durante las últimas décadas las metodologías clásicas han sido reemplazadas por las técnicas moleculares que suplen las carencias de las técnicas anteriormente descritas. Los marcadores de DNA revelan sitios de variación natural a nivel de la secuencia de DNA. Estas variaciones pueden no mostrar cambios en el fenotipo, además los marcadores moleculares no dependen de las condiciones externas, ni del momento del ciclo biológico (Ibáñez et al. 2005). Los primeros marcadores moleculares utilizados fueron los RFLP (Restriction Fragment Lenght Polymorphism) (Tomiç et al. 2013). Esta técnica diferencia entre genotipos, ya que la longitud del fragmento RFLP puede variar como consecuencia de mutaciones puntuales en secuencias de restricción. Se trata de una técnica con una alta reproducibilidad, pero se necesita una cantidad muy elevada de DNA y acaba siendo una metodología costosa y que requiere mucho tiempo. Los RAPD (Random Amplified Polymorphic DNA) siguieron a la metodología anterior. Esta técnica se basa en una reacción PCR (Polymerase Chain Reaction) que utiliza cebadores cortos de una secuencia de nucleótidos arbitraria que resulta en la amplificación de un fragmento de DNA genómico desconocido. Es una técnica relativamente sencilla pero la reproducibilidad entre diferentes laboratorios y las condiciones estrictas experimentales que requiere son difíciles de conseguir, siendo estas las principales desventajas de esta metodología (Weising et al. 2005). Seguidamente, aparecieron los AFLP (Amplified Fragment Lenght Polymorphism), que consisten en las amplificaciones selectivas de fragmentos de DNA generadas por enzimas de restricción, permitiendo el análisis simultáneo de un gran número de loci en un mismo ensayo. Principalmente esta técnica se ha usado para evaluar las

similitudes genéticas entre diferentes variedades y para estudiar las relaciones genéticas entre cultivares de vid (Fanizza et al. 2003).

Actualmente los marcadores microsatélites (SSR) y los SNP (Single Nucleotid Polymorphism) son los marcadores genéticos más utilizados para la caracterización de germoplasma. Los SSR son secuencias cortas de DNA repetidas en tándem, abundantes y dispersas en el genoma. La variabilidad en la longitud de los microsatélites se debe a cambios en el número de unidades de repetición, que son muy fáciles de detectar por PCR. Son de herencia codominante, muy polimórficos, tienen una alta reproducibilidad y la estandarización de la técnica no es excesivamente complicada. Los SNPs se definen como polimorfismos de un solo par de bases de DNA genómico en los que existen diferentes alelos entre los individuos de una o algunas poblaciones. En concreto, en vid, los marcadores SSR son los más utilizados hasta la fecha y han permitido identificar variedades de todo el mundo (Lopes et al. 1999, Lefort & Roubelakis-Angelakis 2001, González-Andrés et al. 2007) así como portainjertos (Sefc et al. 1998, Upadhay et al. 2007, Crespan et al. 2009). Su amplificación se lleva a cabo por PCR y la posterior lectura de los fragmentos generados en la reacción. Los microsatélites han sido desarrollados por varios laboratorios (Thomas & Scott 1993, Thomas et al. 1994, Bowers et al. 1996 & 1999, Sefc et al. 1999, Di Gaspero et al. 2005, Goto-Yamamoto et al. 2006, Arroyo-García et al. 2006). Thomas & Scott (1993) fueron los primeros, identificando 26 variedades de vid cultivada, otras 6 especies del subgénero *Euvitis* y *Muscadinia rotundifolia* utilizando cinco SSRs (VVS1, VVS2, VVS3, VVS4, VVS5). Algunos de estos microsatélites desarrollados han sido seleccionados como grupo de alelos estándar para la identificación de variedades y diferenciación entre ellas al tener un polimorifsmo muy elevado, estos son: VVS2, VVMD5, VVMD7, VVMD27, VrZAG62 y VrZAG79 (This et al. 2004) y son los recomendados por la OIV para identificación varietal (OIV 2009). Con estos seis SSR se considera que es suficiente para poder diferenciar variedades, aunque también se puede incrementar su número. Por ejemplo, en Bowers et al. (1999) se adicionan los marcadores VVMD25, VVMD28 y VVMD32. La utilización de los marcadores SSR además de permitir la identificación varietal, detectando posibles homonimias y sinonimias, es de utilidad para llevar a cabo estudios de variabilidad y establecer relaciones de parentesco. Este tipo de marcador sin embargo no es el más adecuado para distinguir entre clones muy similares de una misma variedad (Pelsy 2010). Los marcadores SNPs también se están

utilizando para la integración genética y física de mapas e inferencia paterna (Anderson & Garza 2006, Lijavetzky et al. 2007), y para estudiar la estructura genética y la historia de la domesticación de la vid (Myles et al. 2011). Estos marcadores son muy abundantes en el genoma de la vid, baratos y fáciles de leer e interpretar, por lo que el intercambio de datos entre laboratorios resulta fácil (Zinelabidine et al. 2012).

Las técnicas más novedosas, actuales y sensibles para el genotipado de vid son el empleo de arrays y microchips (González-Agüero et al. 2013), y el genotipado por secuenciación (GBS; Genotyping by Sequencing), todavía no muy utilizadas por no ser asequibles para todos los laboratorios debido a su elevado coste.

1.4 Principales problemas en el cultivo de la vid

Los principales problemas que presenta el cultivo de la vid incluyen estreses de tipo biótico o abiótico (López-Cortés et al. 2010). Este cultivo está expuesto a muchos estreses causados por hongos, bacterias, fitoplasmas y virus (Laimer et al. 2009). Entre los hongos que pueden causar grandes pérdidas económicas y problemas serios para el cultivo de la vid, encontramos el oídio (*Erysiphe necator*), la antracnosis (*Elisinoe ampelina*), el mildiu (*Plasmopara viticola*) o la podredumbre gris (*Botyris cinerea*) (Wilcox 2011). Otras enfermedades importantes son producidas por bacterias como *Xylophilus ampelinus* que puede empeorar el estado de salud del viñedo y provocar grandes pérdidas en la cosecha (Serfontein et al. 1997); *Agrobacterium tumefaciens* biovar 3 (*Agrobacterium vitis*) causante de las agallas de la corona, induciendo tumores que pueden necrotizar la planta y, la *Xylella fastidiosa* causante de la enfermedad de Pierce. Esta bacteria es muy virulenta y puede matar el viñedo en uno o dos años (Janse & Obradovic 2010). En cuanto a los virus, la vid representa un huésped importante, pues al menos 60 distintos han sido descritos como infectivos para este cultivo (Martelli 2003). Afortunadamente no todos ellos causan mermas en su crecimiento y producción.

Al principio de los años 60, distintos países y la por entonces Comunidad Económica Europea, se involucraron en definir una regulación legal del estado sanitario de la vid, el 86/93/EEC. Teniendo en cuenta aquellos virus que causan mayores daños y de acuerdo con la Comisión directiva 2005/43/EC por la que se modifican los anexos del

Consejo Directivo 68/193/EEC, el material de propagación de vid ha de estar libre de los siguientes virus: virus del entrenudo corto (GFLV), virus del mosaico del arabis (ArMV), virus del jaspeado (GFkV), virus del enrollado 1 (GLRaV-1) y virus del enrollado 3 (GLRaV-3). Entre las medidas de control para evitar la expansión de los virus está el uso de material de propagación sano, pues en campo no hay ningún tratamiento o cura (Bota et al. 2014) y el control de propagación de los vectores de transmisión de dichos virus (Padilla & Martínez 1988). En España, la incidencia de virus en la vid es bastante alta (Bertolini et al. 2010) siendo el GFLV el de mayor prevalencia (95.8%), seguido del GLRaV-3 (94.7%), GLRaV-1 (66.3%) y GFkV (65.3%). El ArMV solo ha sido registrado en Galicia en el verano de 2007 y en primavera de 2008 en la Rioja (Abelleira et al. 2010). La eliminación de las cepas afectadas ha sido, de momento, suficiente para evitar su propagación. Es de gran interés contar con reservorios de plantas sanas libres de virus que sirvan como material madre de propagación y también el mantenimiento en bancos de germoplasma de plantas libres de virus, especialmente de aquellos cultivares que se encuentren en peligro de extinción, y que puedan reintroducirse en campo o en ensayos de mejora cuando se requiera.

1.5 Técnicas útiles para el saneamiento, multiplicación, conservación y mejora de la vid

1.5.1 Técnicas útiles para el saneamiento

Dentro del cultivo *in vitro* existen distintas metodologías para sanear material infectado por virus que se resumen a continuación. La elección del tipo de tratamiento depende de la habilidad de movimiento de las partículas virales en los tejidos de la planta, pues no todos los virus se encuentran en el mismo sitio en la planta ni la velocidad de multiplicación es igual para todos ellos.

1.5.1.1 Termoterapia

La termoterapia consiste en mantener las plantas, o parte de ellas, a temperaturas entre 35ºC y 54ºC, dentro de los límites de tolerancia de cada especie, durante un período de tiempo determinado. El rango más utilizado es el de 35-38ºC, pero en la práctica, la temperatura seleccionada tiene que ser aquella que consiga un equilibrio entre la

degradación del virus y la supervivencia de la planta, por tanto, una correcta elección de temperatura es esencial, pues es un paso limitante (Spiegel et al. 1993). El fenotipo de las plantas puede verse afectado tras un tratamiento con termoterapia, pero parece que es reversible (Basler & Brugger 1981, Greenan & Valat 1992), mientras que los daños producidos por los virus no lo son (Spiegel et al. 1993).

Las alteraciones principales que se producen en el virus con el tratamiento por termoterapia a más de 35ºC están relacionadas con la ruptura de los puentes de hidrógeno y disulfuro de las proteínas de la cápside viral, seguido de una ruptura de los enlaces covalentes fosfodiéster de los ácidos nucleicos. Consecuentemente, la capacidad de infección del virus se ve reducida, se inactiva la replicasa viral, se producen cambios en el pH, se incrementan las enzimas líticas y el RNA viral y mensajero compiten entre ellos por los enlaces ribosomales (Panattoni et al. 2013). La termoterapia ha resultado eficaz para eliminar virus de localización parenquimática (Meng et al. 2017).

Bovey (1958) fue el primero en utilizar la termoterapia en vid. Hizo una revisión de los virus que la infectan y describió el primer protocolo que consistía en mantener las plantas en una cámara de aire a 37ºC durante varias semanas para eliminar el GFLV. Obtuvo una desaparición puntual de los síntomas, pero no una cura duradera. El tratamiento con termoterapia alternando temperaturas, permitió la eliminación de los virus GFLV y ArMV en plantas desarrolladas a partir de ápices de pequeño tamaño cultivados *in vitro* (Monnette 1986).

El principal inconveniente de este tratamiento es que se produzca solo la inactivación y no la muerte del virus, por lo que al establecer nuevamente las condiciones normales de temperatura éste volvería a multiplicarse y a recuperar su concentración. Técnicas combinadas de termoterapia y cultivo de ápices *in vitro* han resultado exitosas en la eliminación de varios virus en diferentes variedades (Maliogka et al. 2009, Pannatoni & Triolo 2010).

1.5.1.2 Quimioterapia.

Es una técnica que permite limitar la incidencia de una virosis mediante el empleo de productos químicos. El desarrollo del tratamiento con quimioterapia no es tan extenso

como el de la termoterapia, pero ha habido varias investigaciones que han contribuido a un mejor entendimiento en la aplicación de esta metodología en vid. Todo empezó con el descubrimiento de la Ribavirina, (Sidwell et al. 1972, Huffman et al. 1973) y hoy en día contamos con más de 40 moléculas antivirales sintetizadas y disponibles en el mercado, pero son pocas en las cuales existe una dosis que permita actuar sobre el virus sin dañar excesivamente la planta (Weiland 2001). Los antivirales se añaden al medio de cultivo y tienen un efecto negativo en la multiplicación del virus, bloqueando el mecanismo de replicación (Kummer & Semal 1970). Generalmente se produce este mismo efecto nocivo sobre la multiplicación celular, por lo que la planta también puede verse afectada.

Monette (1983) utilizó la técnica de quimioterapia en cultivo *in vitro* en ápices de la variedad 'Limberger' (de unos 2mm) cultivados en medios de cultivo con diferentes productos químicos: DHPA (S-9-2,3-dihidroxipropiladenina), Vidarabina (9-β-D-arabinofuranosiladenina) y Ribavirina (1-β-D-ribofuranosil-1-2-4-triazol-3-carboxamida) pero solo se obtuvo material sano utilizando Ribavirina. Panattoni et al. (2005, 2006, 2007a & 2007b) utilizaron diferentes compuestos de tipo nucleosídico y no nucleosídico para intentar eliminar el GLRaV-3 y el Grapevine vitivirus A (GVA); con Tiazofurin consiguieron eliminar el GLRaV-3 y mezclando DHPA y Ribavirina rengeneraron un 40% de plantas libres de GVA. Weiland et al. (2004) intentaron regenerar plantas sanas de vid a partir de explantes infectados con GFLV en la variedad 'Zalema', obteniendo plantas libres de virus solo en el tratamiento con Ribavirina. En Skiada et al. (2013) han publicado el primer resultado satisfactorio sobre la eliminación de GRSPaV (Grapevine rupestris stem pitting-associated virus) usando compuestos antivirales. No obstante, en general, el uso de esta técnica para la eliminación de virus combinados no ha dado resultados satisfactorios, pues los porcentajes obtenidos de plantas sanas no es muy elevado (Guta et al. 2010).

1.5.1.3 Cultivo de meristemos

El cultivo *in vitro* permite el cultivo de pequeñas partes de una planta (explantes) en un medio artificial, estéril y en condiciones adecuadas para que éstas puedan crecer y desarrollarse en plantas completas (Hollings 1965). El cultivo de meristemos consiste en la disección e incubación del meristemo apical de la planta en condiciones de asepsia y ha resultado útil para el saneamiento de plantas infectadas con virus. Se

piensa que, puesto que el meristemo apical es una zona en continua división donde no llegan los haces vasculares, la mayoría de los virus no la pueden alcanzar. El cultivo de esta pequeña parte de la planta puede aislarse y cultivarse para obtener una planta completa que estaría libre de virus. Una segunda teoría de por qué se sanea mediante esta vía, es la inhibición de la replicación vírica en la zona meristemática por la alta tasa metabólica que presenta este lugar y su elevada concentración de reguladores, aunque, tanto los ápices como los meristemos no siempre están libres de virus (Weiland 2001). El cultivo de meristemos es una técnica muy utilizada que en algunos casos requiere de varios meses para obtener nuevas plantas. La adición de reguladores de crecimiento se puede utilizar para favorecer el desarrollo, aunque en algunos casos pueden producirse cambios no deseados como consecuencia de la variación somaclonal (Salazar & Jayasinghe 1997). Esta técnica, se puede combinar con termoterapia (Hatzinikolakis & Roubelakis-Angelakis 1993, Milkus et al. 2000).

1.5.1.4 Embriogénesis somática

La totipotencialidad celular de las células vegetales permite la regeneración de un organismo completo a partir de las células en cultivo (Pierik 1987). La embriogénesis somática consiste en la inducción de embriones a partir de las células de los explantes (hojas, anteras, ovarios, peciolo o incluso embriones zigóticos) dando lugar, posteriormente, a la formación de un organismo completo. La embriogénesis somática es la vía común de regeneración adventicia en vid y se utiliza en este cultivo con distintos fines como la selección de variación somaclonal en medios con distintos tipos de agentes estresantes (Kuksova et al. 1997) o la introducción de genes por transformación genética (Saporta et al. 2016). Mullins & Srinivasan (1976) fueron los pioneros en el uso de esta técnica para la regeneración de plantas sanas de vid a partir de plantas infectadas con virus. Posteriormente, se ha demostrado su eficiencia eliminado varios virus de localización floemática como los del grupo GLR (Grapevine Leafroll) (Goussard et al. 1991) y nepovirus, como el ArMV (Borroto-Fernández et al. 2009) y el GFLV (Gambino et al. 2009). En estos trabajos se han utilizando anteras y/o ovarios como material de partida. También las semillas han sido utilizadas para sanear plantas de vid (Peiró et al. 2015). Utilizando este tipo de explantes se obtienen mayores rendimientos de plantas regeneradas pero como contrapartida se tienen que seleccionar aquellas que provienen de los tejidos maternos, que muestran el mismo

genotipo a la planta de partida (San Pedro et al. 2017), que también tendría que analizarse en las regeneradas a partir de ovarios y anteras.

1.5.1.5 Crioconservación/Crioterapia

La crioconservación consiste en el almacenamiento de células, tejidos u órganos en nitrógeno líquido (-196ºC) con el propósito de la conservación a largo plazo de germoplasma vegetal (Engelmann 1997). Han sido varios los métodos desarrollados para vid, incluyendo la encapsulación-deshidratación, vitrificación, encapsulación-vitrificación y gotita congelada (Yin et al. 2012, Benelli et al. 2013, Bettoni et al. 2016, Bi 2017). La exposición a tan bajas temperaturas produce daños por congelación por lo que se hace necesario una preparación y un tratamiento adecuado antes de la crioconservación en nitrógeno líquido (Engelmann 1997, Wang et al. 2014). La crioconservación se ha descrito como una metodología útil para la erradicación de virus en vid (Pathirana et al. 2015, Bettoni et al. 2016), consiguiendo el saneamiento de plantas infectadas por GVA (Wang et al. 2003), GFLV (Markovic et al 2015) y GLRaV-1, GLRaV-2 y GLRaV-3 (Pathirana et al. 2015). Wang et al. (2003) fueron los primeros en emplear esta técnica utilizando meristemos y dos procesos criogénicos, la encapsulación-deshidratación y la vitrificación para la eliminación del GVA en la variedad 'Bruti'. Los resultados obtenidos indicaron que la erradicación del virus ocurre al congelar en nitrógeno líquido, pues no se obtuvieron plantas saneadas en meristemos que no habían sido previamente congelados. Tambien demostraron que el tamaño del meristemo es importante para una correcta regeneración, pero que no afectaba a la frecuencia de eliminación de virus. Este trabajo fue una buena base para el comienzo del saneamiento de virus a través de la crioconservación.

1.5.2 Micropropagación

La vid es un cultivo que se propaga y multiplica de forma vegetativa y la micropropagación puede representar una técnica alternativa a los métodos convencionales de propagación de plantas (Engelmann 2009) y/o utilizarse en programas de mejora (Alderete et al. 2006). La micropropagación consiste en la multiplicación *in vitro* de pequeños explantes de plantas de forma rápida, eficiente, en masa y en condiciones estériles (Beyl & Trigiano 2014). Si el protocolo de

micropropagación es eficiente, el número de plantas que se obtienen en condiciones controladas y en poco espacio puede ser muy elevado. La micropropagación de vid no es muy común, aunque se han descrito algunos trabajos (De Carvalho-Silva et al. 2012, Dev et al. 2016). En el marco de esta tesis, hemos desarrollado un protocolo de microprogación utilizando como material de partida planta sana cultivada *in vitro* de la variedad 'Monastrell' (San Pedro et al. 2017b).

1.5.3 Conservación *in vitro*

Existen dos formas de conservación de germoplasma:

1. Conservación *in situ*: en el propio lugar donde se cultiva aquello que se desea conservar.
2. Conservación *ex situ*: colecciones en bancos de germoplasma donde se pueden almacenar plantas en campos o parcelas de cultivo o almacenar semillas, polen, DNA o plantas en cultivo *in vitro* o criopreservadas.

La conservación de vid es importante, pues como se ha comentado anteriormente, este cultivo ha sufrido una gran pérdida de variabilidad desde la crisis filoxérica que asoló los viñedos europeos en el siglo XIX. Desde ese momento, todas las variedades cultivadas en Europa se injertaron sobre pies americanos resistentes a la plaga y muchas variedades históricas dejaron de utilizarse. Actualmente se sigue produciendo erosión genética, pues se han ido sustituyendo variedades tradicionales por otras comerciales y son reducidas aquellas incluidas en cada D.O. Además, el impulso de los trabajos de selección sanitaria y clonal, también reducen la variabilidad dentro de una misma variedad, eligiendo solo los mejores clones que son los que después se introducirán en un cultivo (Martínez de Toda 1991).

Existen distintas colecciones de plantas de vid que mantienen extensos conjuntos de germoplasma en campo, por ejemplo 'The Domaine de Vassal' en Montpellier (Francia), el 'Julius Khün Institute' en Siebeldingen (Alemania), y 'La colección de vides de El Encín' en el IMIDRA (Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario) en Alcalá de Henares (España) pero no existen actualmente colecciones de vid en bancos *in vitro.* Es por ello necesario el desarrollo de metodologías de conservación en esta especie, pues con cada material que

desaparece, se pierden genes que podrían tener interés para afrontar diferentes tipos de estrés y adaptarse a nuevas circunstancias.

La conservación *in vitro* consiste en el cultivo de plantas o explantes en condiciones de asepsia controladas en medios de cultivo adecuados y en cámaras de cultivo con temperatura, humedad y fotoperiodo controlado. En este tipo de conservación se necesitan ajustar las condiciones de cultivo para los materiales y subcultivos frecuentes para evitar su envejecimiento o muerte (Cubero 2013). Es por eso interesante evaluar distintas condiciones de cultivo y buscar las más óptimas, de manera que se reduzca el crecimiento de la planta sin afectar a su desarrollo y se minimicen los costes. El crecimiento se puede ralentizar alterando el régimen de iluminación, la composición del medio o la temperatura individualmente o en combinación (Engelmann 1991). En el marco de esta tesis se ha desarrollado un medio de cultivo que ha resultado óptimo para el cultivo *in vitro* de numerosas variedades de vid, y evaluado distintas condiciones para alargar el tiempo entre subcultivos sin perjudicar al crecimiento de la planta y así seleccionar aquellas más adecuadas para la conservación de cada variedad (San Pedro et al. 2018).

Dentro de la conservación *in vitro* de vid, también se desarrollan protocolos de crioconservación (Markovic et al. 2015, Pathirana et al. 2016) que son útiles para la conservación a más largo plazo de duplicados y que complementan con la conservación *in vitro* a corto y medio plazo y con la conservación *in situ*. En el Capítulo 4 también se comentan los ensayos y resultados preliminares que se han abordado en nuestro grupo con este fin.

1.5.4 Mejora genética y transformación genética

El aumento constante de la población y la creciente demanda de alimentos que se necesitan para sostenerla, ha hecho que se necesiten más materias primas, así como una mayor cantidad alimentos por unidad de superficie cultivable. En estos últimos años, la mejora genética en especies cultivadas de plantas ha alcanzado notorios resultados obteniendo nuevas variedades con características y rasgos superiores a las variedades tradicionales (Arterburn et al. 2009). La mejora genética tradicional utiliza comúnmente la introgresión de genes de resistencia u otros genes de interés desde especies donadoras a receptoras utilizando cruzamientos controlados o la mutación

(Gray et al. 2005, Jacobsen & Schouten 2007). Sin embargo, el amplio periodo de tiempo que requieren estos programas y la alta heterocigosidad que presenta la vid limita su uso en este cultivo. Con la mejora genética se espera contribuir a una mayor productividad agrícola, pero esto no se puede llevar a cabo solo con el potencial genético de las variedades de vid, sino que se necesitan obtener cultivares que estabilicen su producción a través de resistencias o tolerancias (Richards 1997). Por tanto, el uso de la transfomación genética en vid es de gran interés, ya que se trata de una herramienta con un gran potencial para introducir genes específicos sin modificar sustancialmente el resto del genoma. En este cultivo se han llevado a cabo ensayos con el fin de realizar investigación básica (Agüero et al. 2006, Dutt et al. 2007) y con fines aplicados (Dabauza et al. 2015, Li et al. 2015). Hasta el momento, la transformación genética en vid se ha centrado, principalmente, en la resistencia a estreses bióticos, especialmente a hongos y virus (Jardak-Jamoussi et al. 2009, Dai et al. 2016), aunque también hay trabajos cuyo propósito es el de aumentar el rendimiento de la producción o la tolerancia a estreses de tipo abióticos, particularmente a las bajas temperaturas (Mezzeti et al. 2002, Constantini et al. 2007, Jin et al. 2009). Durante el periodo de tesis se ha colaborado en la realización de una revisión bibliográfica relacionada con transformación genética en vid con el fin de sintetizar los trabajos realizados en este cultivo y discutir los principales factores que han limitado hasta el momento el éxito de la aplicación de esta técnica (Capítulo 5).

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2. Objetivos

El objetivo principal de esta tesis es el desarrollo y aplicación de técnicas biotecnológicas útiles para el saneamiento, multiplicación, caracterización y conservación de germoplasma de vid.

Se han planeado distintos hitos a cumplir dentro del objetivo propuesto:

1. Desarrollo y aplicación de técnicas para la regeneración de plantas libres de virus.

2. Desarrollo de protocolos de multiplicación *in vitro* a partir de planta sana.

3. Recuperación de variedades antiguas y desarrollo de metodologías para su conservación *in vitro.*

En esta tesis también se pretende analizar el estado actual de aplicación de la transformación genética en vid y sus factores limitantes.

3. Capítulos

Capítulo 1

Somatic embryogenesis from seeds in a broad range of *Vitis vinifera* L. varieties: rescue of true-to-type virus-free plants

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Somatic embryogenesis from seeds in a broad range of *Vitis vinifera* L. varieties: rescue of true-to-type virus-free plants

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Background: Somatic embryogenesis is the preferred method for cell to plant regeneration in *Vitis vinifera* L. However, low frequencies of plant embryo conversion are commonly found. In a previous work we obtained from cut-seeds of a grapevine infected with the *Grapevine leafroll associated viruses 1* and *3* (GLRaV-1 and GLRaV-3), high rates of direct regeneration, embryo plant conversion and sanitation. The aim of this study is to evaluate the usefulness of this procedure for regeneration of other grapevine varieties which include some infected with one to three common grapevine viruses (GLRaV-3, *Grapevine fanleaf virus* (GFLV) and *Grapevine fleck virus* (GFkV)). As grapevine is highly heterozygous, it was necessary to select from among the virus-free plants those that regenerated from mother tissues around the embryo, (true-to-type).

Results: Somatic embryogenesis and plant regeneration were achieved in a first experiment, using cut-seeds from the 14 grapevine varieties Airén, Cabernet Franc, Cabernet Sauvignon, Mencía, Merlot, Monastrell, Petit Verdot, Pinot Blanc (infected by GFLV and GFkV), Pinot Gris, Pinot Meunier, Pinot Noir, Syrah, Tempranillo (infected by GFLV), and Verdil. All regenerated plants were confirmed to be free of GFkV whereas at least 68% sanitation was obtained for GFLV. The SSR profiles of the virus-free plants showed, in both varieties, around 10% regeneration from mother

tissue (the same genetic make-up as the mother plant). In a second experiment, this procedure was used to sanitize the varieties Cabernet Franc, Godello, Merlot and Valencí Blanc infected by GLRaV-3, GFkV and/or GFLV.

Conclusions: Cut-seeds can be used as explants for embryogenesis induction and plant conversion in a broad range of grapevine varieties. The high regeneration rates obtained with this procedure facilitate the posterior selection of true-to-type virus-free plants. A sanitation rate of 100% was obtained for GFkV as this virus is not seed-transmitted. However, the presence of GLRaV-3 and GFLV in some of the regenerated plants showed that both viruses are seed-transmitted. The regeneration of true-to-type virus-free plants from all infected varieties indicates that this methodology may represent an alternative procedure for virus cleaning in grapevine.

Keywords: Grapevine, direct and indirect embryogenesis, microsatellites, TDZ, sanitation.

Background

Virus infections are commonly found under field conditions in grapevine worldwide and multiple infections are routinely detected. Pathogenic agents, including 65 viruses, five viroids and eight phytoplasmas have been detected in a large number of infected grapevines [1]. Among these viruses, the most important are *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine fleck virus* (GFkV), *Grapevine leafroll associated virus* (GLRaV) species which cause the 'grapevine leafroll disease' (GLD), and the rugose wood (RW) complex. Virus infections cause large economic losses in grapevine because of reductions in plant vigour, yield and fruit quality [2]. The long-distance spread of grapevine viruses occurs primarily by the propagation of infected plant material. Therefore, to produce certified material, plants free of the most dangerous viral pathogens are needed. It essential the selection of virus-free plants or the sanitation of virus-infected plants to produce certified material and conserve germplasm for future necessities. Some viruses are particularly recalcitrant to elimination, so therefore different approaches have been tested for virus sanitation [3]: meristem culture [4, 5]; meristem culture combined with thermotherapy [6, 7], chemotherapy [8, 9], and somatic embryogenesis [3, 10-12].

Somatic embryogenesis consists in the induction of somatic embryos from cells of the explants cultured *in vitro* and is the preferred method for cell to plant regeneration in *Vitis vinifera* L. and its intraspecific or interspecific hybrids. It has been used for micropropagation [13, 14], generation of transgenic plants [15] and virus sanitation [3, 12]. Even though many studies have been carried out on embryogenesis in grapevine, the standarization of the conditions for embryogenesis induction and *in vitro* plant regeneration is still an empirical process because it depends on the genotype, type of explant, composition of the culture medium, physiological status of the donor plant, and culture conditions [16-18]. The most limiting factor in grapevine regeneration through somatic embryogenesis is the low rate of embryo to plant conversion, that is, a high percentage of embryos are not able to develop into normal plants [19-21]. In the work of Peiró et al. [12], additionally to plant sanitation, direct somatic embryogenesis (without callus formation) was achieved with high rates of embryo plant-conversion (starting from cut seeds). The time required to recover plants was also shortened with respect to other protocols.

In the present work, we report the results of two experiments aimed to obtain somatic embryo plants (SE-plants) from a total of 16 grapevine varieties (Airén, Cabernet Franc, Cabernet Sauvignon, Mencía, Merlot, Monastrell, Petit Verdot, Pinot Blanc, Pinot Gris, Pinot Meunier, Pinot Noir, Syrah, Tempranillo, Verdil, Godello, and Valencí Blanc), and rescue virus-free plants from grapevine varieties infected with one, two or three viruses commonly found in grapevine (GFLV, GFkV and GLRaV-3). In the first experiment, the putative influence of thidiazuron (TDZ) and genotype was also studied on both, somatic embryogenesis induction and embryo to plant conversion. Among the SE-virus-free regenerated plants, those regenerated from mother tissue (true-to-type) were selected via SSR genotyping.

Results

Regeneration via embryogenesis in fourteen grapevine cultivars, two of them virus infected

Embryogenesis induction

In a first experiment, the regeneration ability was evaluated from cut-seeds of the grapevine varieties Airén, Cabernet Franc, Cabernet Sauvignon, Mencía, Merlot,

Monastrell, Petit Verdot, Pinot Blanc (infected with GFLV and GFkV), Pinot Gris, Pinot Meunier, Pinot Noir, Syrah, Tempranillo (infected with GFLV) and Verdil, grown on medium containing TDZ at 0.90 (EIM2) or 0.45 µM (EIM2/2). After one month of culture, zygotic germination from cut-seeds was observed in some varieties and media conditions (Fig.1). The germination percentage was estimated (ranging from 2.2%, for variety Merlot cultured on EIM2, to 68.3% for Verdil cultured on EIM2/2) and these plants were then removed. On average, germination was higher for seeds cultured on the medium with higher concentration of TDZ (EIM2) (22.35% vs 11.79%) and did not occur in Airén, Cabernet Sauvignon, and Monastrell cultured on medium EIM2/2. In non-cut seeds (sown as controls), germination was absent in both Murashige and Skoog basal medium (MS) as well as in EIM2. After two months of culture (T2), SE was observed in all the assayed varieties (Table 1). The percentage of embryogenic explants ranged from 18.50%, in Airén, to 65.46%, in Verdil and it was mainly achieved via direct embryogenesis (without callus formation) which was observed in at least 90.0% of the explants of the varieties Cabernet Franc, Mencía, Petit Verdot, Pinot Blanc, Pinot Gris, Pinot Meunier, Pinot Noir, and Verdil (Table 1; Fig. 2A). On the contrary, Airén and Monastrell showed great callus formation, that is indirect embryogenesis (Table 1; Fig. 2B). After three (T3) and four months of culture (T4), the somatic embryogenesis percentage (E) had increased, decreased (new embryos were not observed in explants which had responded in a previous period), or been maintained at similar rates - with respect to the previous period - depending on the cultivar (Table 1). There was no effect of the TDZ concentration on direct embryogenesis (DE); both concentrations were able to induce regeneration from cut seeds at a similar extent. Concerning total embryogenesis, no effect of TDZ was observed at T3 and T4, except at T2, when a higher percentage of total embryogenesis was observed in EIM2 with respect to EIM2/2 (48.30% vs 40.29%, respectively. Data not shown).

Taking into account the data of T2, T3, and T4, the most responsive cultivars were Verdil, Petit Verdot, and Cabernet Franc, followed by Pinot Meunier, which had, on average, around 60.0% of embryogenic explants. The less responsive varieties were Airén and Syrah, with maximum E values of 31.5% and 27.57% at T3 and T2, respectively. As Pinot Blanc and Tempranillo were virus-infected, the percentage of regeneration could be different if seeds from uninfected plants were used. However,

around 40% regeneration was obtained in both varieties which suggests that viruses did not inhibit the regeneration response.

Effects of the cultivars and media were found when the number of embryos per explant was analyzed. Besides, the interaction between the cultivars and media was also significant. Few differences were observed among cultivars in the number of embryos per explant, mainly due to variability in the number of embryos per explant and consequently a high error in the estimation. In addition, whereas in medium EIM2 embryo production was similar at T2 and T3, fewer embryos were obtained in EIM2/2 at T3 with respect to T2. Regarding the interaction, the best responding cultivar was Verdil, which had around 10 embryos per explant when cultured on EIM2 (Table 2) and had explants with more than 20 embryos (Fig. 2C). In this cultivar, the amount of embryos per explant was halved when the TDZ concentration in the medium was reduced (an average of 5.11% in EIM2/2 vs 10.54% in EIM2). A similar result was found in Petit Verdot. Considering the percentages of embryogenic calli, the maintenance of embryogenic ability over four months, and the average number of embryos per explant at T2 and T3, the most responsive genotypes were Verdil and Petit Verdot, followed by Cabernet Franc and Pinot Meunier.

Germination of SE: embryo conversion

In the present work, the induced somatic embryos were able to germinate directly in the induction medium (Fig. 2C). To study the development of somatic embryos, 20 germinated embryos per cultivar (from several explants which showed direct embryogenesis) at T2 and T3 were transferred to individual tubes for growth (Fig. 2D). Different parameters related to plant embryo conversion were measured 20 and 40 days later. After 20 days of culture, a low percentage of embryos with normal aspect were observed in the variety Cabernet Sauvignon. Around 90% of plantlets showed white cotyledons, presence of one cotyledon, or fused cotyledons. On the contrary, normal appearance was observed in all plantlets of Pinot Gris and Pinot Meunier. For the rest of the varieties, normal plantlets were in the range of 50-90% (Table 3). In order to compare the development of embryos, we used an index for shoot development (DI) - which takes into account the percentage of plantlets with leaves and the mean number of leaves per plantlet having leaves - and another index for rooting (RI), based on visual root development (Table S1). The highest and the lowest DI values correspond to the cultivars Pinot Meunier and Cabernet Sauvignon,

respectively. The highest RI value was noted for the Verdil cultivar, differences among the other grapevine varieties were scarce. In Fig. 2D, we can see rooted plantlets of the variety Petit Verdot, at different development stages, after 30 days of culture. Along the culture period, some plants that seemed abnormal at 20 days of culture had developed into normal plants at 40 days of culture. This occurred in all the cultivars with the exception of Syrah and Airén, which had a similar embryo conversion (EC) values at both periods. After 40 days of culture, EC was: 100% in Cabernet Franc, Pinot Gris, Pinot Meunier, Tempranillo, and Verdil; 80-95% in Merlot, Pinot Blanc, and Pinot Noir; 50-75% in Airén, Monastrell, Petit Verdot, and Syrah; and around 30% in Cabernet Sauvignon (Table 3). Whereas at 20 days of culture, differences in DI were observed between Pinot Meunier and the rest of the genotypes, at 40 days of culture there were no differences in DI among this variety and Tempranillo, Merlot, and Cabernet Franc. Similar to the results showed above, Pinot Meunier was more developed than the other Pinot cultivars (Table S1).

In addition, flow cytometry analysis demonstrated that all the plantlets obtained by this procedure had the same DNA content as the mother plants, meaning they were diploid.

Rescue of virus-free plants and SSR analysis

The results of real time RT-PCR to detect virus presence in SE-plants from Tempranillo and Pinot Blanc are shown in Table S2. Of 16 Tempranillo SE-plants, 11 were GFLV-free, representing 69% sanitation. With respect to Pinot Blanc, all of the eight plants analyzed were free of GFkV (100% of virus-free plants) and one SE-plant remained infected with GFLV (88% sanitized). The SSR profile by analyzing six SSRs (Table S2) indicates that two SE virus-free plants of Tempranillo and one SE virus-free plant of Pinot Blanc were true-to-type. Therefore, around 10% of the plants were sanitized and displayed the same allele profile as the mother plant.

Virus sanitation of the virus-infected grapevines varieties Cabernet Franc, Merlot, Godello, and Valencí Blanc

Embryogenesis was induced in cut-seeds cultured on EIM2 (Table 4) in explants from all four cultivars each infected with two viruses (Table 5). Mean rates of regeneration indicated that Valencí Blanc was the lowest responding variety of those assayed in

this experiment. As occurred in the previous assay, direct embryogenesis was found with high rates in Cabernet Franc and Merlot whereas indirect embryogenesis predominated in Valencí Blanc and Godello (Table 4). After germination of SE, SE-plants were analyzed in order to assess ploidy and to detect the putative presence of the viruses detected in the mother plants. All SE-plants showed similar DNA-ploidy pattern to mother plants (data not shown) but different sanitation rates were obtained depending on viruses and the variety. All the analyzed SE-plants were GFkV-free. However, the presence of GLRaV-3 was found in Merlot SE-plant, and GFLV in three Godello and one Merlot SE-plants. Therefore, 100% of sanitation was obtained in Cabernet Franc and Valencí Blanc, and ca 80% in Merlot and Godello. These results together with those obtained in the first experiment suggest that the most difficult virus for sanitation among those analyzed is GFLV. The detection of GLRaV-3 and GFLV in SE-plants confirmed the presence of these viruses in the seeds used as starting explants.

Results of SSRs analysis in the regenerated plants (Table S3) showed that at least one plant virus-free per cultivar was true-to-type. The percentage of true-to-type plants with respect to sanitized plant is on average around 10%, similarly to that found in the first assay. Therefore, this methodology permits the rescue of useful grapevine plants free of GFkV, GLRaV-3 and GFLV, even if mother plants are infected with all of these viruses.

Discussion

The high rates of direct regeneration and plant conversion starting from cut seeds described in our previous work aiming to sanitize the cultivar Valencí Negre [12] led us to evaluate the procedure in other varieties to induce regeneration and estimate the efficiency in virus sanitation. This later implicated the analysis of the regenerated virus-free plants genotypes using SSRs (useful plants were those regenerated from mother tissue).

In the first experiment, seed germination (zygotic) after one month of culture occurred from some cut-seeds. This effect that was not observed by Peiró et al. [12] may be consequence of the germination of undamaged embryos after cutting (germination was not observed in non-cut seeds cultured as controls). Despite germination was not the purpose of this work, this result could be interesting in breeding programs to

reduce the intervals in the progenies evaluations as reported in grapevine by Ramming et al. [22]).

Embryogenesis induction and direct regeneration as reported in Peiró et al. [12], was observed in all the 16 assayed varieties, with high rates in 12 of them. This kind of regeneration is convenient since it may reduce somaclonal variation [23-25]. The most common growth regulators used to induce embryogenesis in grapevine are dichlorophenoxyacetic acid (2,4-D) combined with 6-benzyladenine (6-BA) [16, 26] which generally produce high callus formation. The effect of the concentration of TDZ (reduced by half in EIM2/2) was also evaluated in this experiment to increase the possibilities of embryo induction and/or plant conversion. The concentration of the growth regulator is a key factor in both processes and TDZ, a powerful growth regulator, may interfere in shoot development in excess [27, 28]. The obtained results showed that a concentration of 0.9 µM of TDZ is adequate for embryogenesis induction and plant development in grapevine. However, similar or higher response was obtained in EIM2 medium with respect to EIM2/2 medium. Therefore, EIM2 medium was used for the second experiment where induction of embryogenesis and embryo to plant conversion was achieved in the grapevine varieties Cabernet Franc, Merlot, Godello, and Valencí Blanc.

The fact that high percentages of embryogenic explants were produced after only two months of culture from different parts of cut-seeds in all the varieties indicates that regarding embryogenesis induction this protocol is faster than others, where five [3, 10, 29] or seven months [18] are required. In addition, the lowest E values obtained in the present work (in Airén or Valencí Blanc; E>19%) were higher than those reported in other works that used anthers and ovaries as starting explants. For instance, Martinelli et al. [30] reported 2.0% of embryogenic calli in anther and 14.0% in ovary cultures in the Chardonnay cultivar, while E values from 2 to 7% were reported by Oláh et al. [31] - who used anthers of seven interspecific hybrids (*V. berlandieri* x *V. rupestris* or *V. riparia*). The percentages of embryogenesis obtained in the present work were also higher than those reported from stamens and pistils by Prado et al. [18], who achieved an E value of 23.0% in the Mencía cultivar in the best treatment, and Dhekney et al. [17], who reported E values in the range from 0.4 to 35.0% for the best treatments in Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot Gris, Pinot Noir, and Syrah explants. On the other hand, Oláh et al. [32] did not obtained

embryogenesis in anthers from the varieties Cabernet Sauvignon, Merlot, Pinot Gris, or Pinot Noir when they used medium containing TDZ (0.22 µM) combined with 2,4-D (4.97 µM) and low efficiencies (E <5%) were achieved in a medium with 2,4-D (5 µM) and 6-BA (0.44 µM). In the present work, an E value of 27% was achieved in the variety Syrah and E values in a range from 40 to 59% in Cabernet Franc, Cabernet Sauvignon, Mencía, Merlot, Pinot Gris, and Pinot Noir. Besides, this is the first report of successful embryogenesis induction for the varieties Airén, Monastrell, Petit Verdot, Pinot Blanc, Verdil, Godello, and Valencí Blanc. Among the four Pinot varieties, Pinot Meunier had a higher response than Pinot Blanc, Pinot Gris, and Pinot Noir regarding both E and DE. Data from SSR analysis showed higher variability in this cultivar with respect to the other Pinot varieties, which are considered as mutations at the berry colour locus of the variety Pinot Noir [33, 34].

The final step of the embryogenesis procedure is the conversion of embryos into plants. Few reports have focused on this aspect even though a high percentage of grapevine embryos are not able to develop into normal plants [19-21]. In our work, germination of SE occurred directly in the induction medium and embryos were transferred to growth regulator-free medium to follow their development. Noteworthy, several other works reported the requirement of additional labour-intensive steps to go from embryo to plant. For instance, Martinelli et al. [30] first separated clusters of embryos from the embryogenic calli and placed them in a liquid medium; then, the embryos - with the radicals facing downward - were transferred to a medium with 6-BA and IBA or to hormone-free medium for germination. López-Pérez et al. [26] transferred table-grape embryos to a medium with indoleacetic acid, gibberellic acid, and activated charcoal (AC) and then, after germination took place, transferred them to a half-strength MS medium. Comparing to data reported in the literature, the percentages of EC obtained in the present work were high (in average 80.25% after 40 days of culture). EC values around 48%, 55%, and 73% were reported by Dhekney et al. [17] in the varieties Cabernet Franc, Syrah and Merlot, respectively, and an EC of 13.6% was reported for Cabernet Sauvignon by Ben Amar et al. [21]. In varieties different from those used in this work, Goebel-Tourand et al. [19] failed to exceed 20% conversion whereas for López-Pérez et al. [20] conversion ranged from 42.7% to 63.8%. The fact that all the tested varieties in this study produced plants that were well rooted and developed indicates that the growth medium used was good enough for rooting all of them.

Virus sanitation by induction of embryos from stamens or pistils was reported in grapevine to cure plants from GFLV [29], GLRaV [3, 11] or ArMV [10] virus. In the present work, SE-free plants were obtained from plants infected with at least one of the following viruses: GFkV, GFLV or GLRaV-3. The presence of GFkV was not observed in any of the analyzed SE-plants. This was expected as GFkV was reported as not seed transmitted [35]. However, in our study GFLV and GLRaV-3 were detected in some SE-derived plants by RT-PCR indicating that these viruses were in the seed explants. Whereas GFLV was described to be transmitted by seeds in grapevine [36], to the best of our knowledge this is not reported for GLRaV-3 [35, 37]. Despite this result, the sanitation percentages obtained in this work were high (68-100%) for all tested virus and varieties. This result confirms the usefulness of embryogenesis for virus sanitation and the use of seed as starting explants. Sexual embryos are also common explants for somatic induction in other woody species like oak [38], fraser fir [39] or elm [40]. Since grapevine is highly heterozygous, it is propagated vegetatively to keep the same genetic make-up as the parental material. For this reason, we had to select from among the SE-plants, those regenerated from the coat tissue (mother tissue). In grapevine, egg and central embryo sac cells each fuse with sperm cells giving rise to embryo and endosperm development, respectively, whereas seed coats develop from ovule sporophytic tissue [41]. With this aim, the SSRs developed for identification of grape cultivars [42] and recommended by the OIV were used in the first experiment showing that ca 10% of the SE virus-free plant, for each cultivar, were true-to-type. In the second experiment, we increased the number of SSRs (from six to nine), obtaining similar rates of true-to-type sanitized-plants. The use of SSR is common to identify grapevine varieties as well as determine relationships [43, 44]. In conclusion, around 10% of SE plants were suitable for germplasm storage and/or plant multiplication (sanitized and true-to-type). We cannot compare our efficiency respect to that obtained in works that used ovaries or anthers for grapevine embryogenesis induction and virus sanitation [3, 10, 11, 29, 45] because the true-to-type character of SE-plants was not analysed in any of them. In comparison with other sanitation procedures, like meristem culture, the procedure described in the presented study offers the possibility to collect many starting explants which are easier to manipulate than the meristem. Despite meristem culture is the preferred technology for virus cleansing, embryogenesis may be an alternative to consider. Very small meristems are needed for efficient virus cleansing but sometimes the rescue of virus-free plants is not easy. High amount of meristem from previously

in vitro micropropagated plants are also needed. Although good results have been obtained with chemotherapy for some virus and specific varieties, its success depends on the virus, the toxicity produced by anti-viral chemicals, and the variety [8, 46, 47].

Conclusions

The results obtained in two independent experiments showed that the culture of cut-seeds on media containing TDZ is appropriated for embryogenesis induction and plant conversion in a broad sense of grapevine varieties. Cut seeds have the advantage - with respect to other commonly used explants (ovaries and anthers) - that they are easy to collect and use. In comparison with other protocols of grapevine embryogenesis, this procedure reduces the time needed to regenerate plants. In addition, it was confirmed using molecular markers that some embryos were regenerated from mother tissue, which indicates the suitability of this procedure to be applied for different biotechnological purposes such as virus sanitation validated in this work. The high rates of sanitized regenerated plants facilitate the selection of true-to-type ones. In this work, virus-free and true-to-type plants of Tempranillo, Pinot Blanc, Cabernet Franck, Godello, Merlot and Valencí Blanc were obtained from grapevines with GLRaV-3, GFLV and/or GFkV in single or multiple infection. These results open up the possibility of using this technique for the sanitation of grapevine plants infected with other viruses. With respect to virus transmission, GFkV was not found in any of the analyzed plants which was expected because this virus is not transmitted by seed. However, the presence of GLRaV-3 and GFLV in some of the regenerated plants pointed out that both viruses are seed-transmitted.

Methods

First experiment: Embryogenesis induction in fourteen grapevine varieties, two of them virus infected

Plant material, virus detection and somatic embryogenesis

Grapes of the cultivars Airén, Cabernet Franc, Cabernet Sauvignon, Mencía, Merlot, Monastrell, Petit Verdot, Pinot Blanc, Pinot Gris, Pinot Meunier, Pinot Noir, Syrah, Tempranillo, and Verdil were collected in the summer of 2014 in an experimental field ‘Campo de experiencias El Rebollar’ belonging to the ‘Instituto Tecnológico de Viticultura y Enología’ sited at Requena (Valencia, Spain). Asymptomatic leaves

from all the varieties were used to analyze putative virus infection through the methodology described by López-Fabuel et al. [48]. Briefly, extracts were prepared from leaves 1/20 w/v in PBS (Phosphate-Buffered Saline) buffer, pH 7.2, supplemented with 0.2% diethyldithiocarbamic acid (DIECA), and 2% polyvinylpyrrolidone-10 (PVP-10) in individual plastic bags with a heavy net (Plant Print Diagnostics, Valencia, Spain). Total RNA was extracted from 200 µl of crude extract using an Ultraclean Plant RNA isolation kit (Mobio, Carlsbad, CA, USA) following the manufacturer's instructions. The Real-time multiplex RT-PCR was performed for the simultaneous detection of ArMV, GFLV, GFkV, GLRaV-1, and GLRaV-3 using a StepOne Plus thermocycler (Applied Biosystems) and a reaction mixture containing 1x AgPath-ID One-step RT-PCR buffer (Ambion) and 1.5x AgPath-ID One-step RT-PCR enzyme mix (Ambion); 5 µL of sample; 400 nM of GFLV, ArMV, GFkV, and GLRaV-1 primers; 800 nM of GLRaV-3 primers; and 200 nM of each probe (Table S4). The amplification protocol consisted of an RT step at 45°C for 10 min and a denaturation step at 95ºC for 10 min, followed by 45 cycles of amplification (95ºC, 15 s; 50ºC, 15 s; and 60°C, 60 s). As positive controls viral isolates maintained at the Instituto Valenciano de Investigaciones Agrarias (IVIA) were included. When amplification was observed for a specific virus, it was confirmed by real-time uniplex RT-PCR with the corresponding primers.

Immature seeds were extracted by hand from grapes with a mean weight from 0.65 to 0.85 g (6.0 to 7.0 mm long x 3.0 to 3.5 mm wide), corresponding to stage 33 in Pierce and Coombe [49]. The seeds were surface sterilized and cut transversely, before subculturing them on embryogenesis induction medium 2 (EIM2) [12] - that contains salts and vitamins of the McCown Woody plant medium (DUCHEFA, The Netherlands), 4% sucrose, 0.01% polyvinylpyrrolidone-10 (PVP-10), 0.75% plant agar, 0.2% AC, and 0.90 µM TDZ (sterilized by filtration and added to the sterile medium) - or on EIM2/2 medium (equal composition of EIM2 except for TDZ: 0.45 µM). The pH of both media was adjusted to 5.8 before sterilization at 121ºC for 20 min. For each genotype and medium, 10 cut seeds were cultured per Petri dish and 10 repetitions (Petri dishes) per cultivar and treatment were evaluated. While culturing, some plates were removed when contamination was observed and at least eight Petri dishes per culture medium and genotype was used. A sample of non-cut seeds was also cultured on MS and EIM2. The seeds were cultured in darkness for four months, checking the plates monthly. After one month of culture, the percentage of seed

germination (G) was noted and plantlets were removed from the seed. The percentage of seeds with embryogenic explants with DE, and number of embryos per responding explant (NE) were annotated after two, three, and four months of culture (T2, T3, and T4, respectively).

At T2 and T3, for each cultivar, 20 embryos which were initiating germination were transferred to tubes (one embryo/tube) with W medium [50] that contains Lloyd and McCown Woody plant salts, 2% sucrose, 0.01% PVP-10, 0.75% plant agar; supplemented with 1 µM indolbutyric acid (IBA) for growth under standard conditions: incubation in a growth chamber at 26 ± 2ºC under a 16 h photoperiod with cool white light provided by Sylvania cool white F37T8/CW fluorescent lamps (90 µmol m^{-2} s^{-1}). Embryos from each variety were selected from different seeds with direct embryogenesis, although sometimes 2 or 3 embryos per explant were used. Twenty and 40 days after transferring the embryos to tubes, the percentages of normal plantlets (EC) were noted. An index for plant development (DI) was calculated by multiplying the percentage of plantlets with leaves (expressed as a decimal) by the mean number of leaves per plant with leaves. A qualitative index (RI: 1-3, 1: small roots; 2: main root 1.0-1.5 cm or the presence of some secondary roots, 3: main root > 1.5 cm and/or with many secondary roots) was used for scoring the rooting.

Ploidy, Virus and SSR analysis of SE-plantlets

DNA ploidy in at least eight SE-plants per variety was analyzed by flow cytometry like described by Gisbert [51] using as starting material for nucleus extraction leaves from the plants cultured *in vitro*.

Total RNA was extracted from 16 young leaves of SE-plantlets derived from the grapevine cultivar Tempranillo and eight young leaves of SE-plantlets from the cultivar Pinot Blanc. The Real-time uniplex RT-PCR was performed following the same methodology described previously in mother plants.

DNA was extracted from young leaves of SE-plantlets using DNA plant kit (Qiagen). DNA quality and quantity were assessed using gel electrophoresis and spectrophotometry. A multiplex PCR procedure was performed to amplify VVMD5, VVMD7, VVMD27, VVS2, VrZAG62 and VrZAG79 SSRs as described by This et al. [41]. Briefly, the forward primer of the SSR markers was labelled with one of the

four fluorescent dyes, VVMD5 and VVMD27 used carboxy fluorescein (FAM), VVMD7 used carboxytetramethylrhodamine (TAMRA), VrZAG62 used hexachloro-6-carboxyfluorescein (HEX) and VVS2 and VrZAG79 used 6-carboxytetramethyl rhodamine (ROX). Multiplex PCR was carried out in a total volume 11 µl volume using 1.25 µL of commercial Master Mix PCR Multiplex (Takara Multiplex Hot Short PCR, Takara), 20-40 ng of genomic DNA and labeled multiplexed SSR primers (from 5.5 to 35 µM). The amplification was performed in an ABI 9700 thermocycler, and the amplification conditions were 95°C for 14 min followed by 30 cycles of 95°C for 30 sec, 55°C for 90 sec, and 72°C for 60 sec, and a final extension of 72°C for 30 min. Previously to PCR fragment size determination, multiplex PCR products were previsualized using gel electrophoresis. The electrophoresis was carried out on an ABI 3100 platform (Applied Biosystems, Foster City, CA, USA). For PCR fragment size determinations, 0.13 µl of an internal size standard (GeneSacn^{TM} 500 LIZ, Applied Biosystems, Foster City, CA, USA) was mixed with 1 µl of PCR product and 10.87 µl formamide. The mixture was heated at 94°C for 3 minutes and then cooled within icy water. The size of the SSR fragments was determined with the software packages GeneScan 3.7 (Applied Biosystems).

The microsatellite profile of SE plantlets was compared to microsatellite profile from Tempranillo and Pinot Blanc mother plant (source of seeds explants). Both varieties resulted virus-infected after the virus analysis.

Second experiment: sanitation of the virus-infected cultivars Cabernet Franc, Merlot, Godello, and Valencí Blanc

Leaves from the cultivars Cabernet Franc, Merlot, Godello, were collected in July of 2015 in the experimental field 'El Rebollar' (like in the first experiment) and those of Valencí Blanc in a particular garden at Penàguila (Alicante, Spain) and they were used to analyze virus presence following the same methodology described for the first experiment. Briefly, the sanitary statuses of mother plants were analyzed by real-time multiplex RT-PCR and were confirmed by real-time uniplex RT-PCR. Seeds were collected from the analyzed grapevine varieties and embryogenesis induction was performed by culture on medium EIM2 which contains TDZ at 0.9 µM. After one month of culture embryos that occasionally germinated were removed, and somatic embryos at T2, T3 and T4 were transferred to tubes with W medium for growth. Similarly to experiment 1, the ploidy analysis and the sanitary status of at least 10 SE-

plants per cultivar were analyzed. In this experiment, SSRs VVMD25 (ROX), VVMD28 (HEX) and VVMD32 (FAM) were also included to increase the probability to assign the true-to-type. The PCR conditions were the same described above and the allele size determination was performed using the same software packages. The microsatellite profile of SE plantlets was compared to the microsatellite profile of the mother plants.

Statistical analysis

The data were analyzed using the Statistical Analysis System (SAS) version eight statistical package (SAS Institute Inc., Cary, NC, USA). A two-way factorial analysis was conducted to study the effects of the medium and genotype on the percentages of embryogenic explants (E) and embryogenic explants produced via direct embryogenesis (DE) after two, three, and four months of culture, as well as the plant embryo conversion (EC) - measured as the percentage of embryos with normal cotyledons and apex- and plant development, measured as a shoot development index (DI) and a rooting index (RI), after 20 and 40 days of culture. The interaction of both effects was also included - to analyze the number of embryos per explant (NE) for each cultivar after two and three months of culture, and its average. Differences for all traits among the 14 genotypes were evaluated by analysis of variance (ANOVA) testing, except for variety Mencía which was not included for the plant embryo conversion and plant development measurements due to contamination of some tubes. The significance of the differences was determined by a least significant difference (LSD).

List of abbreviations

GFLV: Grapevine fanleaf virus

GLRaV-1: Grapevine leafroll associated virus 1

GLRaV-3: Grapevine leafroll associated virus 3

GFkV: Grapevine fleck virus.

ArMV: Arabis mosaic virus

TDZ: Thidiazuron

SE: Somatic embryogenesis

GLRaV: Grapevine leafroll associated viruses

RW: rugose wood

MS: Murashige and Skoog basal medium

EIM2: Embryogenesis induction medium 2

E: Percentage of somatic embryogenic explants

DE: Direct embryogenesis

DI: Development index

RI: Rooting index

EC: Embryo conversion

2,4-D: Dichlorophenoxiacetic acid

6-BA: Benzylaminopurine

AC: Activated charcoal

G: seed germination

NE: number of embryos per responding explant

PVP-10: polyvinylpyrrolidone-10

Declarations

Ethics approval and consent to participate

Valencí Blanc was collected in a particular garden at Penàguila (Alicante, Spain). The rest of starting materials were collected in a field from the 'Instituto Tecnológico de Viticultura y Enología' (Servicio de Producción Ecológica, Innovación y Tecnología) in Requena (Valencia, Spain).

Consent for publication

Not applicable.

Availability of data and material

Data supporting our findings is contained within the manuscript and its additional files. Several plants were maintained under *in vitro* culture conditions at the COMAV Institute (Universitat Politècnica de València).

Competing interests

The authors declare that they have no conflicts of interest.

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Authors's contributions

TSP, NG and RP conducted the *in vitro* experiments and the microsatellite analysis and participate in the analysis of the results. AO and RP performed the quantitative RT-PCRs for virus analysis and participate in the discussion of these results. CG conceived and led the research, participated in the *in vitro* assays, discussion of the results, and wrote the manuscript. All authors read and approved the final manuscript.

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Table 1. Percentages of total embryogenic explants (E, %) and embryogenic explants obtained via direct embryogenesis (DE, %) after two (T2), three (T3), and four (T4) months of culture. Starting explants consist of 100 seeds distributed a rate of 10 seeds per Petri dish.

Cultivar	T2		T3		T4	
	E	DE	E	DE	E	DE
Airén	18.50 j	0.50 h	31.50 fg	1.50 g	13.00 g	1.00 f
Cabernet Franc	51.26 bcde	51.09 abc	60.50 abc	59.91 a	64.66 a	64.65 a
Cabernet Sauvignon	28.99 hij	23.79 fg	51.15 cd	50.17 abc	46.26 b	44.06 b
Mencía	43.77 defg	40.09 cde	52.17 cd	48.71 abcd	28.73 def	28.71 cde
Merlot	38.10 ghi	35.30 e	54.71 bcd	50.44 abc	37.82 bcd	37.81 bc
Monastrell	42.00 efg	4.75 h	46.44 de	7.55 fg	42.85 bc	25.20 de
Petit Verdot	55.50 abc	49.00 bcd	64.00 ab	56.00 ab	71.00 a	70.50 a
Pinot Blanc	50.00 bcdef	50.00 abc	30.50 fg	30.50 e	22.00 fg	22.00 e
Pinot Gris	54.94 abcd	54.78 ab	45.24 de	45.17 bcd	25.71 ef	25.70 de
Pinot Meunier	60.50 ab	60.50 a	48.50 de	48.50 bcd	44.50 bc	44.50 b
Pinot Noir	39.00 fgh	39.00 de	39.00 ef	39.00 de	34.50 cde	34.00 bcd
Syrah	27.57 ij	16.88 g	26.29 g	15.70 f	26.76 def	19.39 e
Tempranillo	44.47 cdefg	28.77 ef	48.26 de	39.50 cde	48.90 b	44.21 b

Verdil	65.46 a	60.83 a	70.58 a	56.34 ab	49.71 b	42.34 b
ANOVA						
Cultivar	*	*	*	*	*	*
Culture medium	*	NS	NS	NS	NS	NS
Interaction	NS	NS	NS	NS	NS	NS

Different lower case letters within a column indicate significantly different values (P value<0.05).

NS: P value >0.05.

*: P value < 0.05.

Table 2. Number of embryos per explant (NE) for each cultivar after two (T2) and three (T3) months of culture, and the average values. Starting explants consist of 100 seeds distributed a rate of 10 seeds per Petri dish.

Cultivar	Medium	NE at T2	NE at T3	NE/explant
Airén	EIM2	1.70 ± 0.53 b	1.02 ± 0.30 d	1.36 ± 0.26 cd
	EIM2/2	0.83 ± 0.60 b	1.21 ± 0.10 cd	1.02 ± 0.41 d
Cabernet Franc	EIM2	1.24 ± 0.62 b	1.61 ± 0.57 cd	1.43 ± 0.58cd
	EIM2/2	2.51 ± 0.25 b	3.16 ± 0.21 bc	2.84 ± 0.24 cd
Cabernet Sauvignon	EIM2	1.39 ± 0.39 b	1.51 ± 0.14 cd	1.45 ± 0.23 cd
	EIM2/2	1.14 ± 0.41 b	1.25 ± 0.23 cd	1.19 ± 0.27 cd
Mencía	EIM2	1.34 ± 0.38 b	1.15 ± 0.15 cd	1.24 ± 0.22 cd
	EIM2/2	2.03 ± 0.25 b	1.12 ± 0.25 cd	1.57 ± 0.25 cd
Merlot	EIM2	1.57 ± 0.49 b	1.10 ± 0.65 cd	1.34 ± 0.51 cd
	EIM2/2	2.19 ± 0.34 b	1.99 ± 0.35 cd	2.09 ± 0.32 cd
Monastrell	EIM2	2.75 ± 0.39 b	2.71 ± 0.34 bcd	2.73 ± 0.24 cd
	EIM2/2	2.17 ± 0.58 b	1.25 ± 0.64 cd	1.71 ± 0.61 cd
Petit Verdot	EIM2	2.14 ± 0.27 b	4.15 ± 0.47 b	3.15 ± 0.41 bc
	EIM2/2	1.32 ± 0.40 b	1.79 ± 0.63 cd	1.55 ± 0.55 cd
Pinot Blanc	EIM2	2.05 ± 0.19 b	1.40 ± 0.59 cd	1.73 ± 0.32 cd
	EIM2/2	1.19 ± 0.35 b	2.24 ± 0.30 bcd	1.72 ± 0.32 cd
Pinot Gris	EIM2	1.35 ± 0.12 b	1.12 ± 0.14 cd	1.24 ± 0.12 cd
	EIM2/2	1.20 ± 0.28 b	1.07 ± 0.20 d	1.14 ± 0.26 cd
Pinot Meunier	EIM2	1.78 ± 0.31 b	1.81 ± 0.61 cd	1.79 ± 0.43 cd

	EIM2/2	1.15 ± 0.38 b	1.83 ± 0.66 cd	1.49 ± 0.42 cd
Pinot Noir	EIM2	1.35 ± 0.26 b	1.42 ± 0.35 cd	1.39 ± 0.29 cd
	EIM2/2	1.19 ± 0.22 b	1.36 ± 0.58 cd	1.27 ± 0.36 cd
Syrah	EIM2	1.00 ± 0.25 b	0.94 ± 0.27 d	0.97 ± 0.21 d
	EIM2/2	1.21 ± 0.13 b	1.35 ± 0.45 cd	1.28 ± 0.19 cd
Tempranillo	EIM2	2.20 ± 0.42 b	1.23 ± 0.36cd	1.71 ± 0.31 cd
	EIM2/2	2.12 ± 0.49 b	2.15 ± 0.44 bcd	2.14 ± 0.36 cd
Verdil	EIM2	10.77 ± 0.55 a	10.32 ± 0.25 a	10.54 ± 0.33 a
	EIM2/2	8.72 ± 0.58 a	1.50 ± 0.25 cd	5.11 ± 0.53 b
ANOVA	Cultivar	***	***	***
	Culture medium	*	*	*
	Interaction	***	***	***

Different lower case letters within a column indicate significantly different values (P value<0.05).

*: P value < 0.05.

***: P value <0.001.

Table 3. Percentages of embryos with normal cotyledons (EC: embryo conversion, %). As starting explants, 20 somatic embryos from direct embryogenesis events were sown in individual tubes.

Cultivar	EC 20 days	EC 40 days
Airén	52.09 bc	54.77 cd
Cabernet Franc	80.00 ab	100.00 a
Cabernet Sauvignon	11.11 c	29.37 d
Merlot	75.00 ab	80.00 abc
Monastrell	53.46 bc	67.27 abc
Petit Verdot	66.67 ab	75.00 abc
Pinot Blanc	67.78 ab	94.44 ab
Pinot Gris	100.00 a	100.00 a
Pinot Meunier	100.00 a	100.00 a
Pinot Noir	52.50 bc	81.25 abc
Syrah	61.12 ab	61.12 bcd
Tempranillo	88.89 ab	100.00 a
Verdil	78.75 ab	100.00 a

Different lower case letters within a column indicate significantly different values (P value<0.05).

Table 4: Percentages of total embryogenic explants (E, %) and embryogenic explants obtained via direct embryogenesis (DE, %) after two (T2), three (T3), and four (T4) months of culture, and their average. Starting explants consist of 100 seeds distributed a rate of 10 seeds per Petri dish.

Cultivar	T2		T3		T4		Average	
	E	DE	E	DE	E	DE	E	DE
Cabernet Franc	60.00[b]	57.78[c]	68.89[b]	63.33[b]	73.33[c]	61.11[c]	67.40[b]	60.74[d]
Godello	55.00[c]	11.00[a]	77.00[c]	11.00[a]	70.00[c]	9.00[a]	67.33[b]	10.33[b]
Merlot	54.44[b]	41.11[b]	53.33[b]	51.11[b]	54.44[b]	40.00[b]	54.07[b]	44.07[c]
Valencí Blanc	13.00[a]	2.00[a]	17.00[a]	1.00[a]	29.00[a]	5.00[a]	19.67[a]	2.67[a]

Different lower case letters within a column were significantly different (P value<0.05).

Table 5. Virus status of mother plant and plants from SE (somatic embryos) obtained for each cultivar. Percentages of sanitation and percentages of plants regenerated from mother tissue (true-to-type).

Cultivar	Virus status of mother plants	SE plants analyzed	N sanitized plants	% sanitation	SE plants that remain infected	Plants true-to-type[1]	% true-to-type/sanitized plants
Cabernet Franc	GLRaV-3 and GFkV	15	15	100	0	1	6.7
Godello	GLRaV-3 and GFLV	14	11	79	3 with GFLV	1	9.1
Merlot	GLRaV-3 and GFLV	10	8	80	1 with GLRaV-3 and 1 with GFLV	1	12.5
Valencí Blanc	GLRaV-3 and GFkV	10	10	100	0	1	10.0

Figure Captions

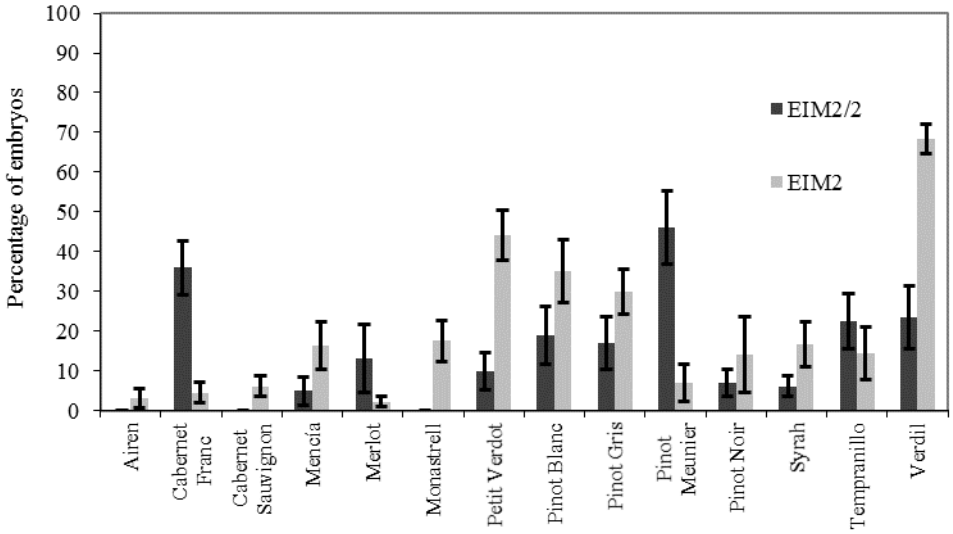

Figure 1. Percentage of germination in cut-seeds (from zygotic embryos) the grapevine varieties Airén, Cabernet Franc, Cabernet Sauvignon, Mencía, Merlot, Monastrell, Petit Verdot, Pinot Blanc, Pinot Gris, Pinot Meunier, Pinot Noir, Syrah, Tempranillo, and Verdil after one month of culture on medium EIM2 (0.9 µM) and EIM2/2 (0.45 µM). Average of 10 plates per variety ± SE.

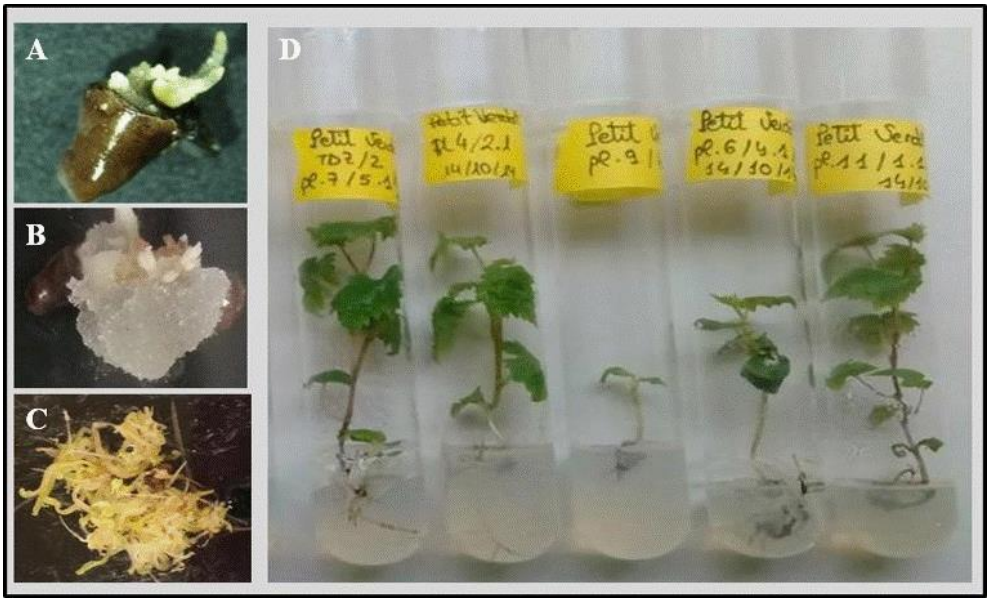

Figure 2. (A-C) Somatic embryogenesis (SE) induction after two months of culture of grapevine explants (cut seeds) on medium EIM2, and plant development: A**.** Direct SE in the variety Pinot Blanc. B**.** Indirect SE in an explant of the variety Monastrell. C. Somatic embryos in an explant of the variety Verdil. D**.** Rooted plants of variety Petit Verdot, 30 days after transferring somatic embryos (from the explants after two months of induction) to tubes with W medium. Among them, one plant has delayed development.

Table S1. Plant development measured using a shoot development index (DI) and a rooting index (RI), after 20 and 40 day of culture. As starting explants, 20 somatic embryos from direct embryogenesis events were sown in individual tubes.

Cultivar	DI[a]	RI[b]	DI[a]	RI[b]
	20 days		40 days	
Airén	0.22 bc	2.14 bc	1.23 de	2.74 a
Cabernet Franc	0.56 bc	1.79 bc	3.75 abcd	2.38 a
Cabernet Sauvignon	0.00 c	1.76 c	0.39 e	2.08 a
Merlot	0.30 bc	1.60 c	3.85 abc	2.75 a
Monastrell	0.41 bc	1.71 c	1.73 cde	2.18 a
Petit Verdot	0.37 bc	1.79 bc	2.79 bcde	2.17 a
Pinot Blanc	0.67 bc	2.32 ab	1.65 cde	2.86 a
Pinot Gris	1.04 bc	1.72 c	2.96 bcd	2.04 a
Pinot Meunier	3.84 a	1.73 c	5.66 a	2.20 a
Pinot Noir	0.57 bc	1.83 bc	3.11 bcd	2.26 a
Syrah	0.44 bc	1.89 bc	1.29 de	2.31 a
Tempranillo	1.43 b	2.31 ab	5.29 ab	2.96 a
Verdil	0.23 bc	2.69 a	2.72 cde	2.93 a

[a]DI was calculated by multiplying the percentage of plantlets with leaves (expressed as a decimal) by the mean number of leaves per plantlet with leaves.

[b]RI is a qualitative index (RI: 1-3, 1: small roots; 2: main root 1.0-1.5 cm or presence of some secondary roots, 3: main root > 1.5 cm and/or with many secondary roots) used for scoring the rooting.

Different lower case letters within a column indicate significantly different values (P value<0.05).

Table S2. Virus status and microsatellites (SSRs) profile of mother plants and plantlets from somatic embryos of cultivars Tempranillo and Pinot Blanc plants.

		VVS2		VVMD5		VVMD7		VVMD27		VrZAG62		VrZAG79	
Cultivar	**Virus status of plants**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
Tempranillo (T) mother plant	**GFLV**	**143**	**145**	**238**	**238**	**239**	**253**	**184**	**184**	**196**	**200**	**247**	**251**
SE-T-s2.6	Free	143	145	238	238	239	253	184	184	200	200	247	251
SE-T-s2.9	GFLV	143	145	238	238	253	253	184	184	200	200	247	247
SE-T-s2.28	GFLV	143	145	238	238	239	253	184	184	200	200	247	251
SE-T-s2.31	Free	143	145	238	238	253	253	184	184	200	200	247	247
SE-T-s2.65	Free	**143**	**145**	**238**	**238**	**239**	**253**	**184**	**184**	**196**	**200**	**247**	**251**
SE-T-s2.68	Free	143	145	238	238	253	253	184	184	200	200	247	247
SE-T-s2.89	GFLV	143	145	238	238	253	253	184	184	200	200	247	247
SE-T-s2.92	Free	143	145	238	238	253	253	184	184	200	200	247	247
SE-T-s2.101	GFLV	145	145	238	238	239	239	184	186	196	200	247	247

SE-T-s2.104	Free	143	145	238	238	253	253	184	184	200	200	247	247
SE-T-s2.107	Free	145	145	238	238	239	239	184	186	196	200	247	247
SE-T-s2.125	Free	143	145	238	238	253	253	184	184	200	200	247	247
SE-T-s2.128	GFLV	143	145	238	238	253	253	184	184	200	200	247	247
SE-T-s2.154	Free	143	145	238	238	239	239	184	184	196	196	247	247
SE-T-s3.40	Free	**143**	**145**	**238**	**238**	**239**	**253**	**184**	**184**	**196**	**200**	**247**	**251**
SE-T-s3.41	Free	145	145	238	238	239	239	184	186	196	200	247	247
Pinot Blanc mother plant	**GFLV and GFkV**	**137**	**151**	**230**	**240**	**239**	**243**	**186**	**190**	**188**	**194**	**239**	**245**
SE-PB-s2.61	Free	151	151	240	240	239	243	186	186	194	194	245	245
SE-PB-s2.141	GFLV	151	151	240	240	239	243	186	186	194	194	245	245
SE-PB-s3.58	Free	137	137	230	230	239	239	190	190	194	194	239	239
SE-PB-s3.59	Free	137	151	240	240	239	243	186	190	188	194	239	245
SE-PB-s3.60	Free	**137**	**151**	**230**	**240**	**239**	**243**	**186**	**190**	**188**	**194**	**239**	**245**
SE-PB-s3.152	Free	137	151	230	230	239	243	186	190	188	194	239	245
SE-PB-s3.153	Free	137	151	230	230	239	243	186	190	188	194	239	245

SE-PB-s4.56	Free	137	151	230	230	239	243	186	190	188	194	239	245

GFLV: Grapevine fanleaf virus, GFkV: Grapevine fleck virus. In bold mother plants and SE plants with the same profile to the mother plant.

Table S3. SSR profiles of the varieties Cabernet Franc, Godello, Merlot and Valencí Blanc and those regenerated through somatic embryogenesis

Cultivar	VVMD7		VVMD5		VVS2		VrZAG79		VrZAG62		VVMD7		VVMD25		VVMD28		VVMD32	
Cabernet Franc	179	187	223	237	140	148	246	258	195	205	237	261	239	255	227	235	238	256
SE CF-S1.107	179	179	223	237	140	148	246	258	195	205	237	261						
SE-CF-S1.150	**179**	**187**	**223**	**237**	**140**	**148**	**246**	**258**	**195**	**205**	**237**	**261**	**239**	**255**	**227**	**235**	**238**	**256**
SE-CF-S1.157	179	179	223	237	140	148	246	258	195	205	237	261						
SE-CF-S1.158	179	187	223	237	140	148	246	258	205	205	237	261						
SE-CF-S2.37	179	179	223	237	140	148	246	258	205	205	237	261						
SE-CF-S2.40	179	187	223	223	140	148	246	258	195	205	237	261						
SE-CF-S2.59	179	187	223	223	140	148	246	258	195	205	237	261						
SE-CF-S2.77	187	187	223	237	140	148	246	258	195	205	237	261						
SE-CF-S2.80	179	187	223	237	140	148	246	258	195	205	237	237						
SE-CF-S2.110	179	187	223	223	140	148	246	258	195	205	237	261						
SE-CF-S2.122	179	187	223	237	140	148	246	258	195	205	237	237						

SE-CF-S2.157	179	187	223	237	140	148	246	258	195	205	237	261						
SE-CF-S2.150	179	187	223	237	140	148	246	258	205	205	237	261						
SE-CF-S3.101	179	187	223	237	140	148	246	258	205	205	237	261						
SE-CF-S3.102	179	187	223	223	140	148	246	258	205	205	237	261						
Godello	**183**	**187**	**223**	**235**	**154**	**160**	**250**	**250**	**187**	**187**	**237**	**237**	249	249	235	257	250	270
SE-G-S1.59	187	187	223	235	154	160	250	250	187	187	237	237						
SE-G-S1.101	187	187	223	235	154	160	250	250	187	187	237	237						
SE-G-S1.136	187	187	223	235	154	160	250	250	187	187	237	237						
SE-G-S2.30	**183**	**187**	**223**	**235**	**154**	**160**	**250**	**250**	**187**	**187**	**237**	**237**	**249**	**249**	**235**	**257**	**250**	**270**
SE-G-S2.84	183	187	235	235	154	160	250	250	187	187	237	237						
SE-G-S2.123	183	187	223	235	154	160	250	250	187	201	237	237						
SE-G-S2.145	183	187	223	223	154	160	250	250	187	187	237	237						
SE-G-S3.61	183	187	223	223	154	160	250	250	187	201	237	237						
SE-G-S3.62	183	187	223	223	154	160	250	250	187	187	237	237						

SE-G-S3.115	187	187	223	235	154	160	250	250	187	187	237	237						
SE-G-S3.136	187	187	235	235	154	160	250	250	187	187	237	237						
Merlot	**185**	**189**	**223**	**233**	**140**	**153**	**258**	**258**	**195**	**195**	**237**	**245**	239	249	227	233	238	238
SE-M-S1.37	185	189	223	223	140	153	258	258	195	195	237	245						
SE-M-S2.7	185	189	233	233	140	153	258	258	195	195	237	245						
SE-M-S2.24	185	189	223	233	140	153	258	258	195	201	237	245						
SE-M-S2.46	185	189	223	233	140	153	258	258	195	195	237	237						
SE-M-S2.137	185	189	223	233	140	153	258	258	195	195	237	237						
SE-M-S3.37	**185**	**189**	**223**	**233**	**140**	**153**	**258**	**258**	**195**	**195**	**237**	**245**	**239**	**249**	**227**	**233**	**238**	**238**
SE-M-S3.38	185	189	223	233	140	153	258	258	195	201	237	245						
SE-M-S3.39	185	185	223	233	140	153	258	258	195	195	237	245						
Valencí Blanc	**179**	**187**	**233**	**237**	**136**	**145**	**243**	**247**	**205**	**205**	**241**	**247**	**255**	**255**	**243**	**257**	**254**	**254**
SE-VB-L 1.10	**179**	**187**	**233**	**237**	**136**	**145**	**243**	**247**	**205**	**205**	**241**	**247**	**255**	**255**	**243**	**257**	**254**	**254**

SE-VB-L 2.1	179	179	233	237	145	145	243	247	205	205	241	247
SE-VB-L 2.6	179	179	233	237	136	145	243	247	205	205	241	247
SE-VB-L 3.1	179	187	237	237	136	145	243	247	205	205	241	247
SE-VB-L 3.2	179	179	233	237	136	145	243	247	205	205	241	247
SE-VB-L 4.4	179	187	233	237	145	145	243	247	205	205	241	241
SE-VB-L 5.1	179	187	233	237	136	145	243	247	205	205	241	241
SE-VB-L 7.1	179	187	233	233	136	145	243	247	205	205	241	247
SE-VB-L 10.1	179	187	233	233	136	145	243	247	205	205	241	247
SE-VB-L 10.2	179	179	233	237	136	145	243	247	205	205	241	247

Table S4: Sequences of the forward and reverse primers and, probes used for TaqMan® RT-PCR.

Virus	Primer forward	Primer reverse	Probe
ArMV	5′-TAG CCC TTG TAC TTA TGG CA-3′	5′-TAT TTA AAC AGT TGA TTC CA-3′	5′-TTG TTA GTG AAT GGA ACG GGG TCA-3′
GFLV	5′-GGGACCACTATGGATGGAATGA-3′	5′-TTCGGTGATATGGAGAGCGAAT-3′	5′-AGT GGA ACG GGA CCA C-3′
GFkV	5′-CGAGAACTCTCTTTTCACCTC-3′	5′-CCGGCGTGGATGTAGAG-3′	5′-ACCCTCGCCCTCATGCA-3′
GLRaV-1	5′-ACCTGGTTGAACGAGATCGCTT-3′	5′-GTAAACGGGTGTTCTTCAATTCTCT-3′	5′-ACGAGATATCTGTGGACGGA-3′
GLRaV-3	5′-AAGTGCTCTAGTTAAGGTCAGGAGTGA-3′	5′-GTATTGGACTACCTTTCGGGAAAAT-3′	5′-CAGGTAATAGCGGACTGAGACTGGTGGACA-3′

Capítulo 2

***In vitro* multiplication of the *Vitis vinifera* L. cv. 'Monastrell'**

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In vitro multiplication of the *Vitis vinifera* L. cv. 'Monastrell'

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Abstract

Background: A protocol for micropropagation of the grape (*Vitis vinifera* L.) cultivar 'Monastrell' was developed. Initial plant material was obtained from the sanitary selection of grapevine plants performed by real-time RT-PCR to confirm the absence of *Grapevine fanleaf virus*, *Arabis mosaic virus*, *Grapevine leafroll-associated virus 1*, *Grapevine leafroll-associated virus 3*, and *Grapevine fleck virus*.

Results: The effects of the salt composition (comparing Lloyd and McCown woody plant medium and Murashige and Skoog medium ½ macronutrients) and the growth regulator benzylaminopurine (BAP), at 0 and 8.9 µM, on plant propagation were evaluated using nodes as explants. The most-efficient procedure consisted of bud induction in the medium with Lloyd and McCown woody plant salts and 8.9 µM BAP for 30 days along with elongation in cytokinin-free medium for 60 days, which gave 22 nodes/explant (174 plants/initial plant). A second cycle of propagation in a medium without BAP for another 60 days could give approximately 10,000 nodes, wich can be obtained after an additional two months of culture. All plants acclimatized after the second cycle of multiplication were successfully transferred to soil.

Conclusion: We developed an optimal protocol for *V. vinifera* cv. 'Monastrell' micropropagation, the first described for this cultivar.

Keywords: Mourvedre, Bud induction, Grapevine, Micropropagation, Mineral salts, Node explants, Quality wines, Real-Time RT-PCR, Virus-free grapevine, *Vitis vinifera* micropropagation, Wineries.

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most-important edible fruit crops cultivated worldwide and is mainly used in wineries [1, 2]. The vast majority of quality wines around the world are made from cultivars resulting from natural or deliberate crosses between different varieties belonging to the *V. vinifera* subsp. *vinifera* [3]. To maintain the resulting combination of the distinct genotypes involved in the crosses, which leads to their distinctive characteristics, vegetative propagation is the common method of grape multiplication. The use of *in vitro* culture for vegetative multiplication, termed micropropagation, offers an important alternative to conventional methods of plant propagation [4, 5, 6] and is an important tool to initiate breeding programs [7]. The use of efficient micropropagation protocols will result in the production of numerous plants that can be maintained under controlled conditions in a reduced space until their transfer to the field for growing or grafting.

In grapevine, virus infection is common and affects the yield and fruit quality and therefore may affect wine quality [8, 9]. In addition, incompatibility problems can be acute in infected vines when grafting [10]. Considering the high cost of establishing a vineyard, it is crucial to use the best-available planting material. In this context, propagation from virus-free materials by micropropagation is of great interest because currently propagation of grapevine is performed by wood cuttings. Moreover, multiplication or culture by *in vitro* procedures is of value in the application of techniques such as induced mutation and selection, *in vitro* screening, and germplasm exchange [11]. Despite the usefulness of this technique, micropropagation attempts using grapevine have had limited success [12, 13]. Recently, micropropagation of several *V. vinifera* cultivars has been described: ‘Malagouzia’ and ‘Xinomavro’ by Skiada et al. [14]; ‘Brasil’, ‘Sun Red’, ‘Pinotage’, and ‘Zinfandel’ by De Carvalho-Silva et al. [15], and ‘Pusa Navrang’, ‘Pearl of Csaba’, and ‘Julesky Muscat’ by Dev et al. [16].

The work conducted using *V. vinifera*, interspecfic hybrids, or grape-related species has ilustrated the influence of the genotypes and the salt composition of the culture medium on the micropropagation procedure [14, 16, 17, 18]. Therefore, this work aimed to develop a micropropagation protocol for a selected clone of ‘Monastrell’, confirmed as virus-free, and compare the most common salt compositions used for grapevine: MS 1/2 [17, 19] *versus* Woody (W) plant salts [13, 20]. ‘Monastrell’ is a grapevine cultivar that originated in the Valencian region of Spain, and it is very important in the Alicante designation of origin (D.O.), Spain. This cultivar is also commonly used in seven D.O.s in Eastern Spain (Valencia, Bullas, Almansa, Jumilla, Yecla,

Benisalem-Mallorca, and Pla i Llevant) and in Southern France (Provence), where it is known as Mourvedre. This cultivar is also used to a lesser degree in five other Spanish D.O.s [21]. To the best of our knowledge, there are no micropropagation protocols for this cultivar.

2. Materials and methods

2.1. Plant material, virus analysis, and in vitro culture

The sanitary status of a single asymptomatic plant of cv. 'Monastrell' was evaluated as described by López-Fabuel et al. [22] to test for *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV), *Grapevine leafroll-associated virus-1* (GLRaV-1), *Grapevine leafroll-associated virus-3* (GLRaV-3), and *Arabis mosaic virus* (ArMV). Viral isolates of each of these virus species, mantained in a screened greenhouse at the Instituto Valenciano de Investigaciones Agrarias, were used as positive controls. Data acquisition and analysis were performed usign StepOne Plus 2.0 software. The cv. 'Monastrell' was cultured *in vitro* in basal medium B (Murashige and Skoog salts (½ macronutrients)) plus vitamins (DUCHEFA, The Netherlands) that conatins 0.025 mg/L $CoCl_2.6H_2O$, 0.025 mg/L $CuSO_4.5H_2O$, 36.7 mg/L FeNaEDTA, 6.20 mg/L H_3BO_3, 0.83 mg/L KI, 16.90 mg/L $MnSO_4.H_2O$, 0.25 mg/L $Na_2MoO_4.2H_2O$, and 8.60 mg/L $ZnSO_4.7H_2O$ as micronutrients; 166 mg/L $CaCl_2$, 85 mg/L KH_2PO_4, 950 mg/L KNO_3, 87.86 mg/L $MgSO_4$, and 825 mg/L NH_4NO_3 as macronutrients; 2 mg/L glycine, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, and 0.1 mg/L thiamine HCl as vitamins; 20 g/L sucrose; 7.5 g/L plant agar; polyvinylpyrrolidone (0.1 g/L); and 0.1 mg/L indolebutyric acid (IBA). Afterwards, clones of virus-free plants were obtained and cultured in tubes. The pH of the medium was adjusted to 5.8 before sterilization at 121ºC for 20 minutes. The cultures were incubated in a growth chamber at 26 ± 2ºC under a 16-h photoperiod with cool white light.

2.2. Shoot multiplication: Effects of mineral salts and benzylaminopurine on growth and proliferation

Four 'Monastrell' plants (7-9 cm tall), grown in *in vitro* culture for 45 days and obtained from the initial virus-free plant, were used as the source of nodes. Eight nodes per plant (each bearing a single axillary dormant bud) were obtained and cultured (one node per tube; Fig. 1A) in tubes containing 16 mL of medium B or W [similar to B but with Lloyd and McCown Woody plant salts; (DUCHEFA, The Netherlands): 0.25 mg/L $CuSO_4.5H_2O$, 36.7 mg/L FeNaEDTA, 6.2 mg/L H_3BO_3, 22.30 mg/L $MnSO_4.H_2O$, 0.25 mg/L $Na_2MoO_4.2H_2O$, and 8.6 mg/L $ZnSO_4.7H_2O$ as micronutrients;

72.5 mg/L $CaCl_2$, 471.26 mg/L $Ca(NO_3)2\ .4H_2O$, 170 mg/L KH_2PO_4, 990 mg/L K_2SO_4, 180.54 mg/L $MgSO_4$, and 400 mg/L NH_4NO_3 as macronutrients; 2 mg/L glycine; 100 mg/L myo-inositol; 0.5 mg/L nicotinic acid; 0.5 mg/L pyridoxine HCl; and 1 mg/L thiamine HCl as vitamins] supplemented with 0 or 8.9 µM benzylaminopurine (BAP) (Fig. 1B). On day 30 of culture, explants cultured on media containing BAP were transferred to baby food jars containing medium B or W (depending on their initial medium) (Fig. 1C). The number of sproutings and yield (number of nodes obtained/initial plant, from the eight initial nodes) were measured 30 and 90 d after the start of the initial culture (Fig. 1D). This assay was performed twice.

The Chi-square test was used to analyze the percentage of sprouting at 30 and 90 d of culture. The effect of the media on yield (number of nodes per initial plant after 60 and 90 d of culture) was analyzed using ANOVA. As significant differences were found, the means were separated by a post-hoc Tukey HSD test ($P<0.05$). The Statgraphics program was used for all the analyses.

For a second multiplication cycle, four plants (10 nodes per plant) obtained from the best procedures (the media with W+BAP) were cultured on medium W without cytokinin for another 60 d (Fig. 1E). The percentage of sprouted buds and the number of nodes of the developed plants were noted at the end of this period.

2.3. Acclimatization and growth in greenhouse conditions

Twenty plants obtained after the second cycle of multiplication were acclimatized in pots containing soil and vermiculite (1:1). The plants were covered with a plastic vessel for 1 week and were grown in a chamber with 70-80% humidity, 26 ± 2ºC, and 1160 lx of luminance for 20 d. Then they were transplanted to pots and grown under hydroponic conditions in a greenhouse. A sample of these plants was transferred to the field.

3. Results and discussion

The analysis of the mother plant, a clone of cv. ‘Monastrell’, was performed to confirm the absence of GFLV, ArMV, GFkV, GLRaV-1, and GLRaV-3 in the starting plant material. Only positive controls gave a successful amplification by real-time RT-PCR, whilst the mother plant tested negative for all five viruses. Subsequently, four plants were obtained from this initial virus-free mother plant to use as sources of nodes to determine the effects of the mineral salt composition and/or

BAP addition on 'Monastrell' *in vitro* plant growth and bud induction. The effect of culture medium mineral composition on the *in vitro* culture of grapevine has been reported by different authors [14, 16, 17, 18]. With regard to the addition of cytokinin to the culture medium, which is essential to increase multiplication in micropropagation procedures, the BAP concentration chosen in this study was similar to that used by Alizadeh et al. [23] for the micropropagation of four grape rootstocks (8.9 µM) and by Abido et al. [24] for the grapevine cv. 'Muscat de Alexandria'. In addition, this dose of BAP was reported as adequate with regard to inducing new buds with good development in other species. For instance, Bhatt et al. [25] considered this concentration optimal for five *Alocasia* species; higher concentrations (22.2 or 44.4 µM) induced pale and stunted shoots.

After 30 d of culture (Fig. 1B) in media without cytokinin, bud break was observed in approximately 45% of the explants (precisely, 37.5% of those cultured on B and 50% of those cultured on W), whereas in the BAP-containing media, new bud induction was observed in 87.5% of the nodes cultured on B+BAP and in all the nodes (100%) cultured on W+BAP. The Chi-square test comparing the two media without cytokinin showed no significant difference (P-value=0.78). Similarly, no difference was obtained when comparing the two media with cytokinin (P-value=0.85). Adventitious buds were transferred to media without growth regulators for elongation (Fig. 1C); the remaining nodes were maintained in the corresponding tubes for sprouting or elongation. After 90 d of culture, 62.5% sprouting was achieved on medium B and 87.5% on medium W; no significant difference between media B and W was found (P-value=0.84). For both media with BAP, 100% of the nodes had new shoots.

Adequate elongation of shoots was produced for all treatments (Fig. 1D). In grape, difficulties in shoot elongation [13] or deficiencies such as vitrification [23] in BAP-containing media have been described. Difficulties in shoot elongation or vitrification were not observed during the protocol developed here, possibly because of the use of another genotype, differences in the composition of the culture media, or the transfer of shoots induced in a BAP-containing medium to a medium without growth regulation for elongation.

Yield, measured as the number of nodes obtained from an initial plant after a period of culture, was calculated after 60 and 90 d of initial culture. Statistical differences were found between the media at both times of initial culture (P-value=0.0022 at 60 d and 0.0001 at 90 d). The yield observed from the explants cultured on medium W was approximately double that of explants cultured on medium B, in the absence or presence of BAP, on both days of scoring (Fig. 2). Therefore, it was concluded that medium W is better than B for the *in vitro* growth of the grape cv.

'Monastrell'. The most-efficient multiplication was obtained from nodes cultured on medium W supplemented with BAP and elongated in medium W; 174 shoots - 5-15 cm tall - were obtained from each initial plant (8 nodes) at day 90 of the initial culture, averaging 21.75 nodes/explant. This result is better than that obtained by De Carvalho-Silva et al. [15] using a lower BAP concentration and similar time of culture for four cultivars of *V. vinifera* (ranging from 1.9 to 2.8 nodes/explant). Medium W has an auxin, indole-butyric acid (IBA), that favors rooting and also contains polyvinylpyrrolidone that may favor rooting induction [26]. Concerning the mineral composition of the media, the main differences were the higher levels of SO_4^{2-}, PO_4^{3-}, and Ca^{2+} and lower NO_3^- in medium W than in medium B. Moreover, the thiamine HCl concentration was ten times higher in medium W.

After the initial propagation step, the number of clones can be increased by using a second cycle of multiplication. Of the 40 nodes (extracted from four plants) obtained in the first cycle of propagation and cultured on medium W without cytokinin for 60 d, 38 shoots sprouted and grew (each with 6.53 ± 0.21 nodes/shoot). Therefore, in this second cycle of multiplication, approximately 62 nodes were obtained per plant (6.53 nodes/shoot x 38 shoots/4 plants). Considering that we obtained 174 plants in the first cycle, approximately around 10,000 nodes (174 plants x 62 nodes/plant) could have been produced to start a third multiplication step.

Finally, the acclimatized plants were 8.4 ± 0.40 cm tall, on average, 20 d after transplanting. All the plants transferred for growing under greenhouse and field conditions were adapted (Fig. 1G-I).

In conclusion, the salt composition of medium W doubled the yield with respect to medium B, with and without the addition of BAP. By following the most efficient micropropagation procedure of those tested (nodes of the mother plant cultured on medium W containing 8.9 µM BAP for 30 d; then, transfer of the induced buds to medium W for elongation for 60 d and a second cycle of multiplication in medium W for another 60 d), approximately 10,000 clones of cv. 'Monastrell' rooted plants that can be transferred to soil with high efficiency could be obtained from one initial plant after approximately seven months of culture.

Conflict of interest

The authors declare no conflict of interest.

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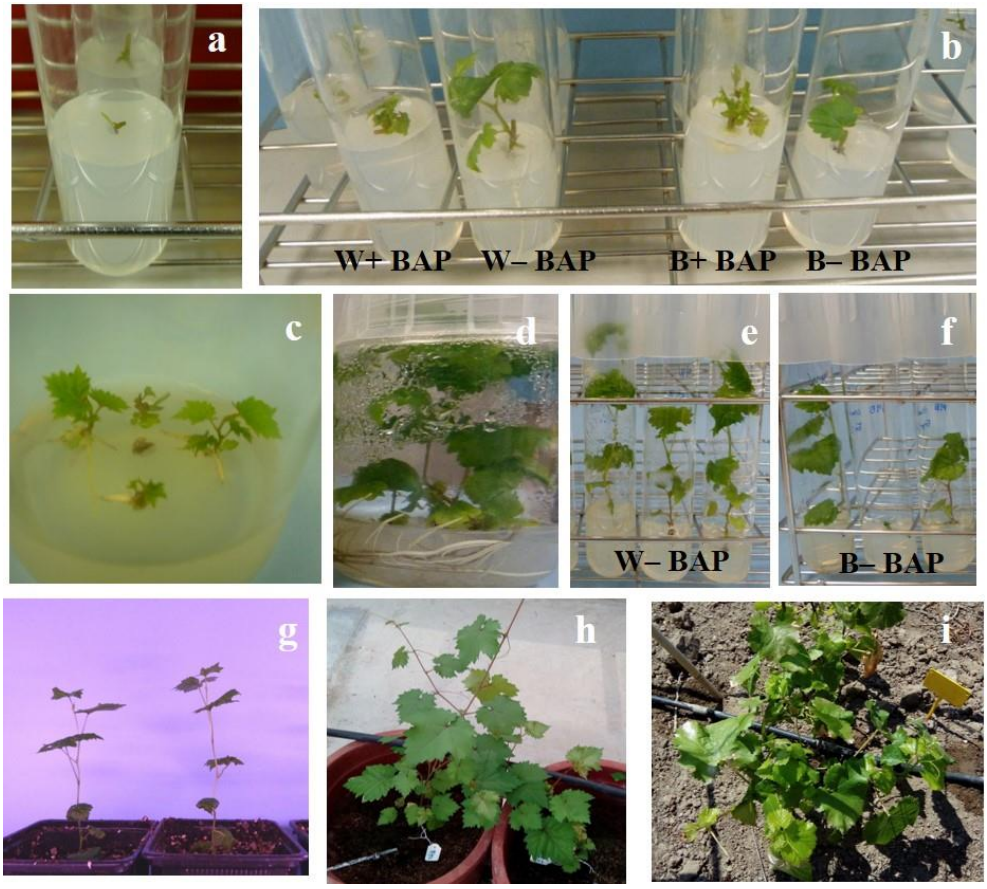

Fig. 1. (a) Nodes on culture media at day 0. (b) Explants grown on medium W or B supplemented or not with BAP (8.9 μM) after 30 d of culture. (c) Shoots induced in BAP-containing media and developing in media without growth regulators. (d, e, f) Shoots developed after 90 d of culture. (g) Plants, 15 d after acclimatization. (h, i) Micropropagated plants grown under greenhouse (h) and field conditions (i).

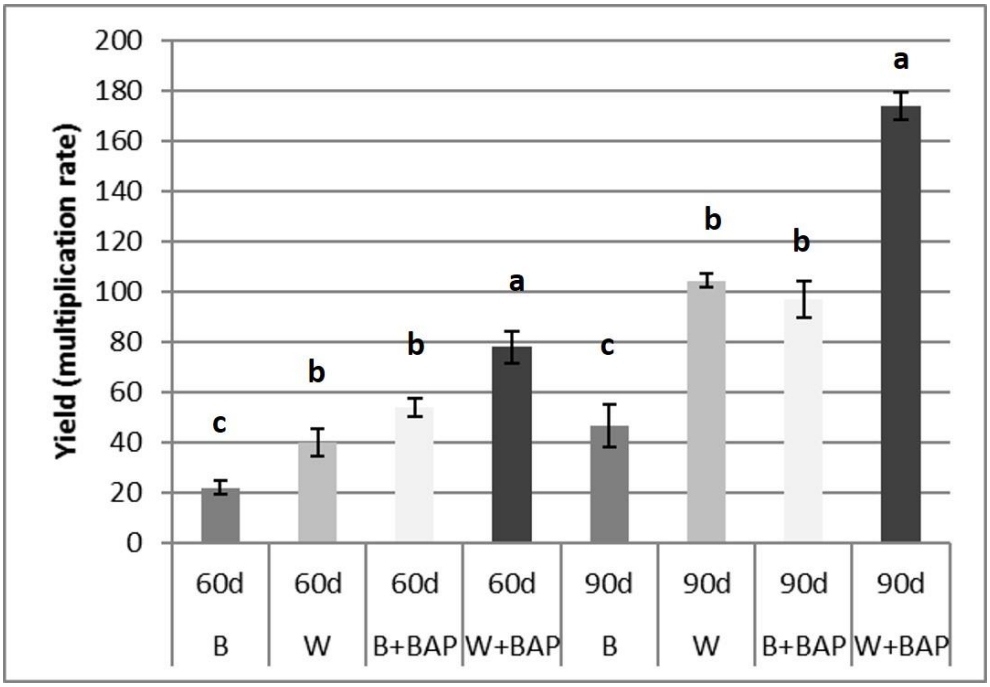

Fig. 2. Mean values of the propagation rate (yield) after 60 and 90 d of culture on media B and W, supplemented or not with BAP (8.9 μM). Yield: number of nodes obtained per initial plant after a period of culture. Mean values separated by different letters are significantly different ($P < 0.05$) according to Tukey's test.

Capítulo 3

Evaluation of conditions for *in vitro* storage of commercial and minor grapevine (*Vitis vinifera* L.) cultivars

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Evaluation of conditions for *in vitro* storage of commercial and minor grapevine (*Vitis vinifera* L.) cultivars

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Evaluation of conditions for *in vitro* storage of commercial and minor grapevine (*Vitis vinifera* L.) cultivars

Abstract

In vitro culture represents a tool for the *ex situ* conservation of a high number of sanitized plants in a reduced space. However, the culture media and/or other growing conditions need to be optimized to minimizing plant growth and storage cost. Growth on MW medium was evaluated in the commercial cultivars 'Airén', 'Bobal', 'Chardonnay', 'Garnacha Blanca', 'Moscatel de Alejandría', 'Moscatel de Grano Menudo', 'Pedro Ximénez', 'Pinot Blanc', 'Pinot Gris', 'Sauvignon Blanc', and 'Tempranillo'; the minor cultivars 'Chelva', 'Valencí Negre', 'Valencí Blanc', and 'Verdil'; and the endangered cv. 'Esclafacherre'. Different growth rates were observed among cultivars: those with faster growth need to be subcultured every 1.5-2.0 months; those with the slowest growth every 3.5-4.0 months. The effect of halving the sucrose in MW reduced the growth of the cultivars that grew faster without compromising survival. When IBA was removed from MW, growth was also reduced in some cultivars. Therefore, small modifications of the MW composition are adequate for grapevine *in vitro* storage under standard incubation conditions. This is an advantage with respect to the change of temperature used in other work to achieve growth reduction, and allows the use of the same chamber for different *in vitro* culture procedures.

Keywords: *Vitis*, conservation, *in vitro*, germplasm, sanitized, endangered

Introduction

In situ and *ex situ* preservation of germplasm are complementary approaches for germplasm storage that are necessary to preserve the genetic diversity of particular species that provide the raw material for breeding programs (Scherwinski-Pereira & Costa, 2010; Vasanth & Vivier, 2011). *Ex situ* preservation in germplasm banks is achieved by maintaining seeds at a low temperature and/or the growth of vegetatively propagated plants under *in vitro* culture conditions. The latter methodology offers a set of advantages that overcome some of the limitations inherent to traditional conservation, such as the culture of healthy plants (virus-free material) in controlled conditions and the storage of a high number of plants in a reduced space (Ray & Bhattacharya, 2010). In addition, it facilitates the interchange of materials among different germplasm banks or their transference to other users.

Grapevine (*Vitis vinifera* L.), one of the most important fruit crops in the world (Mederos, 2007; Mukherjee, Husain, Misra, & Rao, 2010), includes cultivars grown for fresh or dried fruit

consumption or (mainly) for vinification (Vivier & Pretorius, 2002). Around 10,000 cultivars have been described (Robinson, Harding, & Vouillamoz, 2012). However, only 5,000 cultivars are in current use and 3% of these - the most commonly used, including 'Cabernet Sauvignon', 'Merlot', 'Syrah/Shiraz', 'Chardonnay', and 'Sauvignon Blanc' - dominate vineyards worldwide (Anderson & Nelgen, 2011). Therefore, grapevine is affected by genetic erosion and is losing its initial variability, mainly affecting the oldest material of each region (Martínez de Toda, 1991).

The genetic diversity of grapevine is maintained normally as living plants in the field (Leão & Motoike, 2011; Santana et al., 2008) and there are different *ex situ* collections: for example, 'The Domaine de Vassal' in Montpellier (France), 'Julius Khün Institute' in Siebeldingen (Germany), and 'El Encín' (IMIDRA) in Alcalá de Henares (Spain). All these collections are exposed to the potentially harmful effects of factors like climatic change, pest attacks, and budget constraints (Engelmann, 2009). Therefore, *in vitro* preservation can be a good complement for grapevine storage, as has occurred in other vegetatively propagated crops like banana - which is preserved in the INIBAP germplasm bank, in Leuven (Belgium), through *in vitro* culture storage and cryopreservation procedures (Panis, 2009).

The success of *in vitro* conservation is tightly related to the period of time required for storage and to the choice of an adequate conservation method (García-Águila, De Feria, & Acosta, 2007). Before the start of *in vitro* preservation, it is convenient to check the sanitary status of the plants. Later, it is also germane to provide suboptimal culture conditions that limit and slow down plant development, without causing physiological damage to the plant material, in order to reduce the number of subcultures and hence minimize the cost and the putative errors that could arise in each subculture. To reduce growth, and therefore the number of subcultures, modifications of the *in vitro* culture conditions, such as a decrease of the temperature or light, are commonly used (García-Águila, et al., 2004, Hassanen, Abido, Aly, & Rayan, 2013). *In vitro* sanitation for grapevine started in the middle late of the last century when meristem culture and thermotherapy was applied for virus santitation (Doré & Varoquaux, 2006; Engelbrecht & Schwerdtfeger, 1979). Later, other techniques such as chemotherapy, electrotherapy, cryotherapy, and somatic embryogenesis were reported (reviewed in Panattoni, Luvisi, & Triolo, 2013, from 1991 to 2010; Bayati, Shams-Bakhsh, & Moieni, 2011; Peiró, Gammoudi, Yuste, Olmos, & Gisbert, 2015). The first attempts to store grapevine under *in vitro* culture conditions were reported by Barlass & Skene (1983), Alleweldt (1985) and Galzy (1985). However, field collections have played the preeminent role in grapevine conservation. Recently, interest has been raised in developing protocols for storage of grapevine under *in vitro* culture (De

Carvalho-Silva, Gomes-Luis, & Scherwinski-Pereira, 2012; Hassanen et al., 2013) or cryopreservation (Bettoni, Dalla Costa, Pereira Gardin, Kretzschmar, & Pathirana, 2016; Marković, Chatelet, Sylvestre, Kontić, & Engelmann, 2013).

The aim of this work was to evaluate the ability of 16 grapevine cultivars (including commercial, minor, and endangered cultivars) to grow on MW medium under standard incubation conditions and to establish modifications to this medium to reduce the growth of the faster growing cultivars, with the aim of lengthening the subcultures and minimizing the storage costs. The germplasm used was confirmed as virus free for Grapevine fanleaf virus (GFLV), Grapevine leafroll-associated virus 1 (GLRaV-1), Grapevine leafroll-associated virus 3 (GLRaV-3), Grapevine fleck virus (GFkV), and Arabis mosaic virus (ArMV).

Material and methods

As a first step, the following 11 grapevine (*V. vinifera* L.) cultivars were employed: 'Airén', 'Chardonnay', 'Chelva', 'Garnacha Blanca', 'Moscatel de Alejandría', 'Moscatel de Grano Menudo', 'Pedro Ximénez', 'Pinot Blanc', 'Pinot Gris', 'Sauvignon Blanc', and 'Verdil'. All of them produce white berries. 'Chelva' was described as a cultivar for table consumption, and with long storage, whereas 'Verdil' was used for vinification; both are considered historic cultivars of Valencian origin (Janini, 1922).

Clones were obtained from one plant of each cultivar, previously established *in vitro* and confirmed as virus free for GFLV, GLRaV-1, GLRaV-3, GFkV, and ArMV by real time multiplex RT-PCR, performed as described by López-Fabuel et al. (2013). Plants were cultured in MW medium (described by Peiró et al., 2015) containing 2.46 g l^{-1} of Lloyd & McCown Woody Plant Medium including vitamins, DUCHEFA, The Netherlands (Lloyd & McCown, 1980), 20 g l^{-1} sucrose, 0.1 g l^{-1} polyvinylpyrrolidone-10 (PVP-10), and 7 g l^{-1} plant agar. The pH was adjusted to 5.8 before autoclaving for 20 min at 121ºC and a pressure of 1 atmosphere; 0.2 mg l^{-1} of indole-butyric acid (IBA) was added after autoclaving, when the culture medium had cooled. The conditions in the *in vitro* chambers were: 70% humidity, a temperature of 25 ± 1ºC, and a photoperiod of 16-h (achieved using Sylvania Agro-Lux F36W/CRO-tubes).

Evaluation of plant development in the standard MW medium

Between 10 and 12 nodal explants (1 cm long approximately and each containing an axillary bud) per cultivar were cultured in glass tubes (1 per tube) containing MW. After 40 days of growth the following variables were estimated: 1) Sprouting percentage (number of sprouted explants x 100/number of total explants); 2) Explant length: cm of sprouted bud; 3) Percentage of plants with green leaves (number of plants with leaves x 100/number of sprouted explants); 4) Number of leaves per plant; 5) Root development (on a scale of 0-3: detailed in Figure 1A-D); and 6) Percentage of plants with callus in the roots (number of plants with callus in the roots x 100/number of sprouted explants: see Figure 1E).

In a second assay, the cultivars 'Tempranillo' and 'Bobal', commonly used for vinification in the Valencia DO (Designation of Origin), the minor cultivars 'Valencí Negre' and 'Valencí Blanc', of Valencian origin (Janini, 1922; Rojas-Clemente, 1809), and the endangered cultivar 'Esclafacherre' (VVAA, 1899) were also cultured on MW to determine growth rates, rooting ability, and putative callus formation. All three are grown for table consumption and can be used for vinification.

Study of the effects of the growth regulator IBA on growth in MW

The effects of the elimination of IBA from the MW culture medium were evaluated in cultivars exhibiting more than 50% callus formation in MW at 40 days of culture. Growth rates, rooting ability, and putative callus formation were estimated at 40 days of culture.

Study of the effects of a decreased sucrose supply on growth in MW

The effects of a 50% reduction in the sucrose concentration in the MW culture medium were evaluated in cultivars showing fast growth (plant length ≥ 4 cm in MW at 40 days of culture). Growth rates, rooting ability, and putative callus formation were estimated at 40 days of culture.

Statistical analysis

The model used to evaluate the effect of MW medium on plant length, root development, and the number of leaves at 40 days of growth included the cultivar (with 11 levels) effect. To analyze the effect of the growth regulator IBA, the culture medium effect (two levels; 0.0 and 0.2 mg l^{-1} IBA), the cultivar effect (five levels), and the double interaction at 40 days of growth were included. The same model was employed to evaluate the effect of sucrose effect in the culture medium (two levels; 10 and

20 g l^{-1} sucrose) on six cultivars. A Bayesian analysis was performed. Bounded flat priors were used for all unknowns. The data were assumed to be normally distributed. Marginal posterior distributions of all unknowns were estimated using Gibbs Sampling. After some exploratory analyses, we used one chain of 1,000,000 samples, with a burning period of 200,000. One sample each 100 were used in order to avoid high correlations between consecutive samples; thus, marginal posterior distributions were estimated with 8,000 samples each. Convergence was tested for each chain using the Z criterion of Geweke (Geweke, 1992).

Results and discussion

In vitro culture is a good methodology that complements field storage (Engelmann, 2009) but has not been commonly applied for *Vitis* species. A first evaluation of growth ability (sprouting, growth, and rooting) in MW medium, previously selected for propagation of cultivar 'Monastrell' (San Pedro, Villanova, Peiró, Olmos, & Gisbert, 2017), was performed for 11 cultivars checked as virus-free for GFLV, GLRaV-1, GLRaV-3, GFkV, and ArMV through real time multiplex RT-PCR, as described by López-Fabuel et al. (2013). Plants free of these five viruses are required in the EU Directive 2002/11/EC rules for propagation. The tested cultivars included the commonly used commercial cultivars 'Airén', 'Chardonnay', 'Garnacha Blanca', 'Moscatel de Alejandría', 'Moscatel de Grano Menudo', 'Pedro Ximénez', 'Pinot Blanc', 'Pinot Gris', and 'Sauvignon Blanc', and the minor cultivars 'Chelva' and 'Verdil'.

Differences in growth among cultivars under *in vitro* culture conditions are expected because genotype is a key factor in *in vitro* culture. In *Vitis* species, the influence of genotype has been reported for propagation (Mukherjee et al., 2010) and rooting (Lewandowski, 1991). Table 1 shows data for growth (sprouting rates, percentages of plants with leaves, and plant length) and rooting (callus formation and root development). Sprouting was high: 100% in cultivars 'Airén', 'Chelva', 'Moscatel de Alejandría', 'Moscatel de Grano Menudo', 'Pedro Ximénez', 'Pinot Blanc', 'Pinot Gris', 'Sauvignon Blanc', and 'Verdil'; 92% in cultivar 'Chardonnay'; and 70% in cultivar 'Garnacha Blanca' (Table 1). Developed leaves were visible in more than 80% of plants of each cultivar, with the exception of 'Garnacha Blanca' (57%); this cultivar and 'Moscatel de Grano Menudo' grew the slowest (with mean lengths of 1.04 and 2.01 cm, respectively) while 'Pedro Ximenez' and 'Verdil' (7.62 and 5.68 cm, respectively) grew the fastest. With respect to rooting, all the cultivars were able to root (100%) despite the fact that callus formation was observed in seven of the 11 cultivars, in the range 33-100% (Table 1, Figure 2B). The average root development value was higher than 1.0 for all cultivars: this indicates that they rooted appropriately in this medium and implies that the probability

that the value of the root development stage of these cultivars was higher than 1 (a relevant value for this trait) was at least 50%. The time required for subculture was 3.5-4.0 months for cultivars that grew slowly and 1.5-2.0 months for those with faster growth.

We consider MW medium as adequate for the storage of grapevine cultivars in *in vitro* culture, with a minimum number of subcultures when the plant length is less than 4.0 cm after 40 days of initial culture. Therefore, it could be used to maintain 'Garnacha Blanca', 'Moscatel de Alejandría', 'Moscatel de Grano Menudo', 'Pinot Blanc', and 'Pinot Gris', since the probability that their lengths exceeded 4 cm was lower than 30%. Modifications of MW medium to lengthen the subculture times for cultivars 'Airén', 'Chardonnay', 'Chelva', 'Pedro Ximénez', 'Sauvignon Blanc', and 'Verdil' (the probability that their plant lengths exceeded 4 cm was at least 60%) were tested: concretely, the effects of halving the sucrose concentration in MW ($MW_{s1/2}$) and IBA elimination. By reducing growth, the number of subcultures needed for germplasm maintenance is reduced, thus minimizing the costs.

Lowering the sucrose supply did not affect the sprouting of any cultivar (data not shown) but reduced plant growth (length) in cultivars 'Airén', 'Pedro Ximénez', and 'Verdil'. For cultivars 'Airén' and 'Pedro Ximénez' root development was reduced, although they maintained the proper root development stage (mean value higher than 1.0; Figure 3B). The overall average height, considering all the cultivars, was less than 4 cm. Therefore, $MW_{s1/2}$ medium seems to be suitable for *in vitro* culture since it slowed down plant development without impeding rooting. The probability that the length of these cultivars in $MW_{s1/2}$ medium exceeded 4.0 cm was lower than 10% (Figure 3A).

When grown on the medium without IBA (MW0), callus formation was not observed in any of the cultivars that produced callus in MW, namely 'Chardonnay', 'Chelva', 'Moscatel de Alejandría', 'Pinot Gris', and 'Sauvignon Blanc' (Table 1). Probably, these cultivars had higher contents of auxins than the cultivars that did not show callus, and suffered an excess when grown in MW. Callus formation was also reported by Mukherjee et al. (2010) in *Vitis champinni*, a consequence of adding the auxin IBA (0.3, 0.5, and 0.7 mg l^{-1}) to the culture medium (60.5, 72.1, and 82.3% callus formation, respectively). In other work, the presence of auxin in the culture medium was reported as essential for the good rooting of *V. vinifera* and *V. labrusca* (De Carvalho-Silva et al., 2012; Heloir, Fournioux, Oziol, & Bessis, 1997; Lazo-Javalera et al., 2016). However, in the cultivars evaluated in the present study, rooting was produced in MW that lacked IBA, although the development of roots was poorer than with MW, especially in 'Moscatel de Alejandría' (Figure 2B). Plants of this cultivar and of 'Chelva', 'Pinot Gris', and 'Sauvignon Blanc' showed, in these media, average lengths of 2 to

3 cm (at 40 days of culture). Therefore, MW0 could be appropriate for the *in vitro* storage of these three cultivars, with subcultures every 3.5-4.0 months.

Similar height and root development stage in MW medium was observed for a same cultivar in the different assays, which indicated that there are no relevant differences among batches. All the assayed cultivars are maintained under the selected conditions since nowadays.

The cultivars 'Tempranillo', 'Bobal', 'Valencí Negre', 'Valencí Blanc', and 'Esclafacherre' were able to root without callus and grow in standard MW, needing subcultures about every two months. Growth in media with half the normal concentration of sucrose and with or without IBA is under evaluation, the aim being to extend the time of subculture.

Conclusion

The MW culture medium is adequate for the sprouting, growth, and rooting in *in vitro* conditions of all the evaluated germplasm, which includes the commercial cultivars 'Airén', 'Chardonnay', 'Garnacha Blanca', 'Moscatel de Alejandría', 'Moscatel de Grano Menudo', 'Pedro Ximénez', 'Pinot Blanc', 'Pinot Gris', 'Sauvignon Blanc', and 'Tempranillo'; the minor cultivars 'Chelva', 'Verdil', 'Bobal', 'Valencí Blanc', and 'Valencí Negre'; and the endangered cultivar 'Esclafacherre'. 'Garnacha Blanca' had the lowest growth rate. A decrease in the sucrose content of the MW medium diminished the growth of the cultivars that grew faster ('Airén', 'Chardonnay', 'Chelva', 'Pedro Ximénez', and 'Verdil'), lengthening the subcultures. The exclusion of IBA from the MW medium resulted in the inhibition of the callus formation that was apparent in 'Chardonnay', 'Chelva', 'Moscatel Alejandría', 'Pinot Gris', and 'Sauvignon Blanc' in MW. Therefore, MW medium, with or without simple modifications, is sufficient to maintain - at reduced costs - the assayed *V. vinifera* cultivars under standard *in vitro* culture conditions, in a single growth chamber.

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Disclosure statement

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Table 1. Sprouting (%), plants with leaves (%) plants with callus (%), and length (cm), number of leaves per plant, and root development stage [High Posterior Density at 95%], for the 11 white grapevine cultivars in MW culture medium at 40 days of culture.

Cultivar	Sprouting (%)	Plants with leaves (%)	Plants with callus (%)	Length (cm) [$HPD_{95\%}$]	Number of leaves/plant [$HPD_{95\%}$]	Root development stage [$HPD_{95\%}$]
Airén	100	100	83	5.44 [4.09;6.77]	4.44 [3.27;5.60]	2.04 [1.79;2.32]
Chardonnay	92	91	64	4.64 [2.90;6.07]	5.02 [3.56;6.25]	1.55 [1.20;1.89]
Chelva	100	100	100	4.17 [2.74;5.49]	3.74 [2.52;4.92]	2.41 [2.10;2.73]
Garnacha Blanca	70	57	0	1.04 [0.05;2.34]	1.43 [0.06;2.76]	1.05 [0.69;1.41]
Moscatel de Alejandría	100	90	70	2.48 [1.00;4.15]	3.10 [1.65;4.56]	1.93 [1.60;2.29]
Moscatel de Grano Menudo	100	100	0	2.01 [0.70;3.44]	3.18 [1.99;4.38]	1.77 [1.53;2.10]
Pedro Ximénez	100	100	0	7.62 [5.74;9.36]	5.27 [3.77;6.76]	2.83 [2.50;3.17]
Pinot Blanc	100	92	33	3.72 [2.63;5.33]	4.52 [3.50;5.66]	1.94 [1.70;2.24]
Pinot Gris	100	83	58	3.27 [1.84;4.43]	3.53 [2.33;4.85]	1.60 [1.35;1.95]
Sauvignon Blanc	100	90	70	4.01 [2.18;5.52]	6.08 [4.81;7.40]	2.32 [2.03;2.72]
Verdil	100	92	0	5.68 [4.10;7.13]	5.79 [4.38;6.95]	2.26 [1.97;2.58]

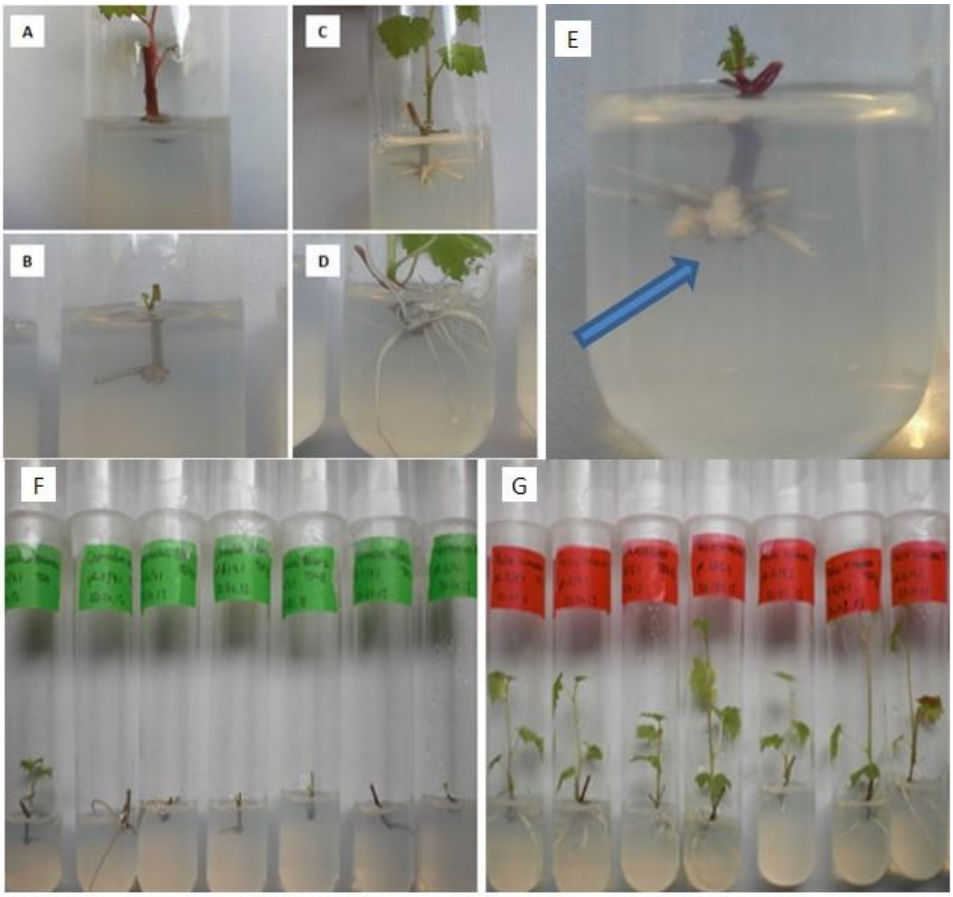

Figure 1. Root development **(A)** stage 0, **(B)** stage 1, **(C)** stage 2, **(D)** and stage 3. **(E)** Root with callus (arrow). Development of the cultivars **(F)** 'Garnacha Blanca' and **(G)** 'Pedro Ximénez' at 30 days of culture in MW.

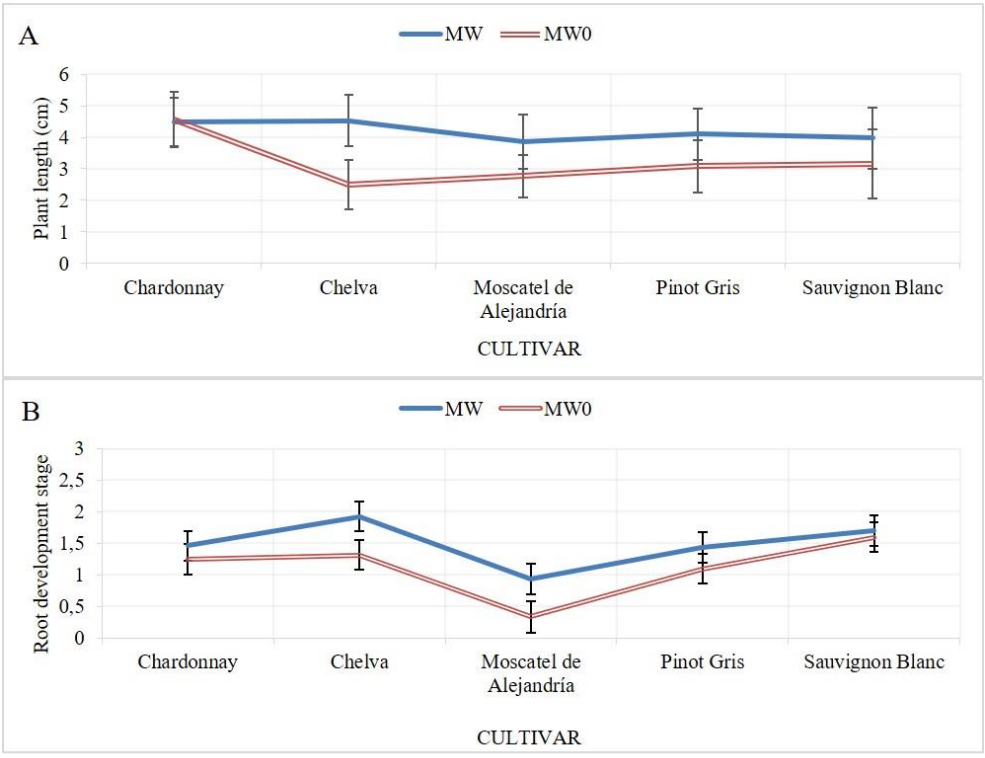

Figure 2. A) Plant length (cm) and **B)** Root development stage (± standard error) at 40 days of culture in MW (blue) and MW0 (red), of the five white grapevine cultivars that had callus in the roots in MW at 40 days of culture in the first assay.

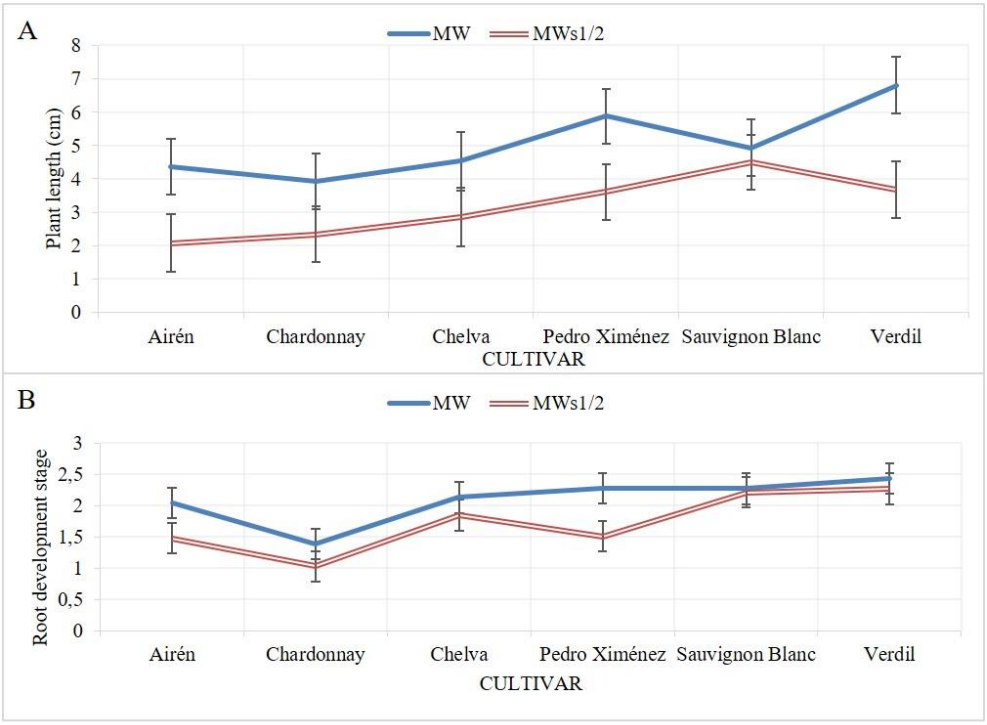

Figure 3. A) Plant length (cm) and **B)** Root development stage (± standard error) at 40 days of culture in MW (blue) and MWs1/2 (red) of the six white grapevine cultivars that showed growth greater than 4 cm in MW at 40 days of culture in the first assay.

Capítulo 4

Recovering Ancient Grapevines Varieties: from Genetic Variability to *In vitro* Conservation, a Case Study. (Capítulo de libro)

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Recovering Ancient Grapevines Varieties: from Genetic Variability to *In vitro* Conservation, a Case Study.

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ABSTRACT
A great number of varieties have been described in grapevine, however few of them are currently in use. The increasing concern on varietal diversity loss has encouraged actions for recovering and preserving grapevine germplasm which represents valuable resources for breeding as well as for diversification in grapevine-derived products. On the other hand, it is expected that this important crop, which is distributed in warm areas worldwide, will suffer the climate changes. Therefore, it is also convenient the identification of intravarietal variability and the recovery of accessions well adapted to particular environments. In this chapter, we will contribute to highlight the importance of recovering ancient materials, the usefulness of SSR markers to determine their molecular profile, the importance to analyze their virus status and the possibilities that offer biotechnological tools for virus sanitation and *in vitro* storage as a complement of field preservation. In this context we have evaluated different grapevine accessions and developed *in vitro* culture protocols for micropropagation, sanitation and storage grapevine cultivars. In this work, we report the results obtained for the historic variety 'Valencí Blanc' (or 'Beba') and the historic and endangered variety 'Esclafagerres' ('Esclafacherres' or 'Esclafacherris').

Keywords: 'Valencí Blanc', 'Beba', 'Esclafagerres', 'Esclafacherres', virus, sanitation, varietal identification, *in vitro* culture

1. Introduction

Grapevine (*Vitis vinifera* L.) is a crop of major economic importance distributed in warm areas worldwide [1] with a wine production of 2,910 million hectoliters in 2014 and 75,866 square kilometers dedicated to grapevine culture [2]. The majority of the world's wine producing regions are found between the temperate latitudes of 30 and 50° in both hemispheres [3]. Grapes are mainly used for making wine, but also can be eaten fresh as table grapes or used for making jam, juice, jelly, grape seed extract, raisins, vinegar, and grape seed oil. Approximately 71% of world grape production is used for wine, 27% as fresh fruit, and 2% as dried fruit. In the VIVC (*Vitis* International Variety Catalogue; http://www.vivc.de/), supported by Biodiversity and the OIV (International Organization of Vine and Wine), there are around 24,500 accessions which include cultivars, breeding lines and different *Vitis* species. Around 50% (12,679) of the varieties correspond to *Vitis vinifera* Linné Subsp. *vinifera* (or *sativa*) and 30% (7,714) correspond to *Vitis* interspecific crossing. Around 25% of the cultivars were registered in France (5,602), follow by United States (2,401) and Italy (2,348) with approximately 10% each one. Spain has registered a total of 734 varieties, being most of them (631) *V. vinifera*. According to Lacombe [4], a total of 1,902 grape varieties (both scions and rootstocks) are officially authorized for cultivation in at least one country of the European Union. Around 65% of these grape varieties are registered only in one country, meaning the responsibility to preserve these varieties are too focused. On the other hand, four varieties (‘Cabernet Sauvignon’, ‘Merlot’, ‘Chardonnay Blanc’, and ‘Sauvignon Blanc’) were maintained in at least 60 different institutions. Nowadays, not only these cultivars but also ‘Syrah’ (or ‘Shiraz’) dominate vineyards worldwide [5]. Considering that most major wine producing regions could become by 2050 unsuitable for currently grown cultivars [6, 7], it is important the preservation of genetic variability and the selection and/or development of cultivars well adapted to upcoming climate changes. The long juvenile period of grapevine makes breeding a slow process, therefore the knowledge of the raw material and their availability are very important to speed up breeding programs.

Grapevine: gain and loss of diversity

Vitis vinifera subsp. *vinifera* was domesticated in the Neolithic period (*ca* 8500-4000 BC) [8] from wild grapevines (*Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi) [9-12]. Grapevine domestication appears to have occurred between the seventh and the fourth millennia BC, in a geographical area between the Black Sea and Iran [8, 13-16] and the earliest evidence for large-scale winemaking was

found to the North of Zagros Mountains and in the Caucasian region around 6000-5000 BC [17]. Cuttings of cultivated grapevines would have been spread by humans in the Near East, Middle East and Central Europe. As a result, these areas may have constituted secondary domestication centers [18, 19] where spontaneous hybridizations among cultivars or local wild plants generated the pattern of admixture that is observed in current cultivars [19-23]. In consequence, genetic variability of grapevine has increased due to the contribution of different genetic pools in the process of grapevine spreading. The appearance of spontaneous mutations [24] and the selective pressure by humans which depended on the different uses of grapevine (fresh consumption, raisin or wine production) [25] were also contributed to increase the genetic variability of this crop.

Along the years, genetic erosion has occurred in both, cultivated and wild grapevines. Anthropogenic pressure on the wild natural habitats greatly decreased the wild grapevine populations that were also affected by the phylloxera aphid (*Daktulosphaira vitifoliae* Fitch) introduced from North America during the second part of the 19th century [26]. The phylloxera pest devastated the vineyards in all Europe. Since that time, grapevines need to be grafted onto phylloxera resistant rootstocks. This fact reduced the number of grapevine cultivars used as scions which provoke genetic erosion [12]. On the other hand, the creation of Denominations of Origin (DO), each one including a reduced number of authorized varieties, has also contributed to reduce the varieties cultured in a specific area. Therefore, the preservation of grapevine minor cultivar and that on risk of disappearance together with V*itis vinifera* subsp. *sylvestris* is a major stake in grapevine preservation.

Grapevine preservation

The importance of germplasm preservation is focused on their putative use, in the present or in the future. It is the source of genes to face new pathogens or climate constraints. Genetic diversity of grapevine is maintained normally as living plants in the field [27, 28]. Several important *ex situ* grapevine collections exist like ‘The Domaine de Vassal’ in Montpellier (France), the ‘Julius Khün Institute’ in Siebeldingen (Germany), and ‘La colección de vides de El Encín’ in the IMIDRA (Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario) center of Alcalá de Henares (Spain). The French collection houses 7,800 accessions of *ca* 50 countries, representing 2,300 different grape varieties, including wild species, rootstocks, hybrids and mutants. Its transfer to the INRA Pech Rouge Experimental Unit (Gruissan, Aude) is under progress (https://www6.montpellier.inra.fr/vassal_eng/). The German collection of the Institute for Grapevine Breeding Geilweilerhof holds more than 3,000 accessions of cultivars and wild species as well as

important breeding lines. Beyond many others old and neglected cultivars from Germany, Switzerland and Austria and rare germplasm from Eastern Europe can be found (https://www.julius-kuehn.de/en/grapevine-breeding/fields-of-activity/genetic-resources-and-information-centre-vine-and-wine/). The Spanish collection consists of 3,532 accessions that are grouped into 852 rootstocks, 69 interspecific hybrids, 111 *Vitis* spp., 1,852 *V. vinifera* varieties, of which 1,178 are for wine use and 674 for table use, and 648 *V. vinifera sylvestris* (http://www.madrid.org/coleccionvidencin/index.php?option=com_content&view=article&id=9&Itemid=2). In order to avoid the loss of the stored materials which are exposed to environmental disasters and pest attacks, the duplication of accessions for storage in different collections are a common strategy, although limited by budget constraints.

Other complementary strategy very useful in vegetative propagated plants is the *in vitro* preservation that offers the possibility to maintain plants under controlled and slow growing conditions and their micropropagation and transference to the field when need it. Although the first attempts to store grapevine under *in vitro* culture conditions were reported in 1980s [29-31], this strategy is not usual in grapevine, although it is commonly used in other vegetative propagated crops like banana (preserved both through standard *in vitro* conditions and cryopreservation in the International Network for the Improvement of Banana and Plantain germplasm bank, in Leuven, Belgium). Cryopreservation is the storage of viable tissues, generally meristems or embryos, at ultra-low temperature [32]. The success of *in vitro* conservation is tightly related to the choice of an adequate conservation method with the development of the corresponding methodology [33].

It is convenient to check by molecular markers that the variety to be stored really correspond to it; in grapevine it is commonly found homonymies (similar name for different cultivars) and/or synonymies (different names for a same cultivar). The identification of homonymies is important to avoid the loss of variability (loss of genotypes). On the contrary, the detection of synonymies avoids the maintenance of duplicated materials that do not contribute to increase variability but increase the cost. There is also very important to check the sanitary status of the plants, sanitize them if necessary, and provide suboptimal culture conditions that limit and slow down plant development, without causing physiological damage to the plant material. Grapevine can be infected by numerous viruses [34] and a high incidence of virus infection is commonly found in autochthonous cultivars [35, 36]. The EU Directive 2002/11/EC rules require that the initial plant material for vegetative propagation is free of *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine fleck virus* (GFkV), *Grapevine leafroll associated virus-1* (GLRaV-1), and *Grapevine leafroll associated virus-3*

(GLRaV-3). When samples to be storage are virus infected, different approaches to regenerated virus free plants can be used. Since the middle late of the 20th century, meristem culture and thermotherapy were applied with this aim for grapevine sanitation [37, 38]. Other techniques such as chemotherapy, electrotherapy, cryotherapy and somatic embryogenesis were also reported [39-41].

For *in vitro* preservation under standard or limiting conditions, it is necessary the development of protocols adjusted to the variety to be preserved. For this kind of storage, it is important to choice the adequate culture medium and environmental conditions in order to reduce the number of subcultures and hence minimize the cost and the putative errors that could arise in each subculture. Protocols for storage grapevine under *in vitro* culture have been reported by several authors [42- 43]. Recently, we reported the effectiveness of the MW medium to store a broad spectrum of grapevine cultivars, including endangered varieties, as well as the modifications of this medium (reduce of sucrose or elimination of indole-3-butyric acid (IBA) in the medium) to reduce growth in the faster growing varieties [44]. Cryopreservation protocols have also been developed for grapevine [45-47]. When developing cryopreservation protocols, the evaluation of the cryopreservation solutions toxicity in the varieties to be preserved is required. Pre-culture in culture media that facilitate the dehydration of tissues (i.e. in medium with high content of sugars) is also a common step.

Finally, studies of genetic variability among varieties under conservation are important because they allow the detection of mutations and the study of relationships among them. The determination of molecular profiles is also of great importance in the development of core collections which represent the higher variability present in a whole collection in a reduced number of accessions. The core collections are very useful for breeders because studies on the core collection provide an overall view of the properties to be found in the whole collection [48]. In the following schema, the different steps to be carried out for germplasm storage are shown (Figure 1).

2. Recovering ancient germplasm at the Comunitat Valenciana: the historic varieties ‘Valencí Blanc’ and ‘Esclafagerres’

Richness of grapevine germplasm in the Comunitat Valenciana

The Comunitat Valenciana which includes the provinces of Alicante, Valencia and Castellón is located in the Mediterranean coast of Spain and has been an important viticulture area since historic times to nowadays. For instance, in the archeological place of ‘L’Alt de Benimaquia’ (Denia,

Alicante), dated back to the end of the seventh century BC, significant quantities of vinification residues (tartaric acid and seeds) were found [49]. Important vineyards must be also located in the Requena-Utiel plateau (Valencia) as evidenced by the big presses dated in the fifth century BC found at Las Pilillas site [50]. Nowadays there exist three DO of origin for wine production (DO Alicante (http://www.vinosalicantedop.org/), DO Valencia (http://www.dovalencia.info/) and DO Utiel-Requena (http://utielrequena.org/)), one DO for table grape (DO Uva de mesa del Vinalopó (http://uva-vinalopo.org/wp/)) and one Protected Geographical Indication in Castellón (http://www.igpcastello.com/).

The richness in grapevine cultivars before the arrival to the phylloxera pest in the provinces of Alicante and mainly, in that of Valencia, is well documented [51, 52]. In a report about grapevines varieties cultured in Spain in 1889, it is pointed that more than 150 varieties were cultured in different locations of the Valencia province. These varieties included varieties with berries of black, white and red color. In comparison with other provinces that also appeared in this report, Valencia was one of the richest [52]. The phylloxera aphid that devastated European vineyards invaded Spain in 1878 from three areas (Girona, Málaga and Portuguese border). Its arrival to the Comunitat Valenciana, with the consequent loss of grapevine variability, occurred in 1912 when the aphid spread to Sagunto, Líria and Requena [53].

On the context of the research project CGL2015-70843-R, we initiated different approaches in order to contribute in the recovering of ancient varieties in risk of disappearance from Alicante and Valencia provinces. The objectives of this project include the analysis of grapevine germplasm diversity, and the development of protocols for virus sanitation and *in vitro* conservation. Different prospections have been performed in order to rescue ancient varieties. The determination of SSR profiles is being useful to confirm the identity and to detect synonymies and homonymies that are very common in grapevine. The analysis of the genetic variability will identify accessions which may carry useful mutations for adaptation to specific environments. As occurred in other areas, grapevine cultivars were found commonly infected by the viruses GFLV, GLRaV and GFkV. Sanitation of cultivars to be preserved *in vitro* is being carried out through meristem culture, although other alternative sanitation procedures are being developed [42, 54]. In this work, we report the SSR profiles of different accessions of the historic variety 'Valencí Blanc' or 'Beba and the historic and endangered variety 'Esclafagerres' (or 'Esclafacherres/is'). The methodologies used for their sanitation and *in vitro* conservation are also summarized.

The historic varieties 'Valencí Blanc' and 'Esclafagerres'

The 'Valencí Blanc' variety also known as 'Beba' is a minor cultivar usually used as white table grape (Figure 2A). Despite in the past it was used for wine and raisin production [52, 55], today is cultured as table grape for minor consume and it is authorized for wine production in DO Ribera del Guadiana (Spain), where it is also named as 'Eva' (http://riberadelguadiana.eu/esp/). The origin of this variety is unknown although it is proposed an oriental or North African origin [56]. The name 'Valencí' ('Valensi' and 'Balansí' in older reports) remembers to the name of Valencia City [57]. Oliver-Fuster (1980) cited by [58] proposed that this variety maybe was introduced by Balearic people who emigrate to Argelia. The most antique synonymy assigned for this variety is 'Calop' [59] although other synonymies like 'Ain el Kelp', 'Tebourbi', 'Panse the Provence' and 'Grumer' are reported [56, 58 - 60]. In a report about grapevines cultivated in 1889 [52], it is noted the culture of 'Grumer', among other 18 grapevine varieties in the Alicante province and in Albaida and Onteniente (locations nearest to the Alicante province). In the same report appeared the culture of 'Valensí' in Alberique and Enguera (nearest to Valencia City). Recently, with the name of 'Grumer', we have identified some accessions from the Alicante province that do not correspond to 'Valencí' but grouped with 'Muskat Istambul' [61]. Lacombe et al. [62] proposed as the origin of 'Muscat Istambul' the cross of 'Muscat of Alexandria' x 'Valencí Blanc' which was confirmed by Mena et al. [63]. Therefore, it could be easy to find this homonym. In the VIVC database appeared 71 synonymies for 'Beba'. However, some of these ('Chelva', 'Hebén', 'Mantúo', 'Teta de Vaca' and 'Uva de Planta') were rejected as did not share the same SSR profile [64]. Probably other of those proposed are also false synonymies. On the other hand, in the report about grapevine varieties cultured in Spain in 1889 [52], the variety 'Valencí' was included in the groups of cultivars with black and white berries. The comparison among the SSR profiles of some accessions of 'Valencí' with white grapes ('Valencí Blanc') and with black grapes ('Valencí Tinto' or 'Valencí Negre' in the Comunitat Valenciana) indicates that they are not mutant for berry colour but resulting from different crosses. Comparing the SSR profile of the variety 'Heben' (or 'Gibi'), proposed as parent of 'Valencí Blanc', with the SSR profile of the accession of 'Valencí Tinto' hold in this database, no relationship between them were observed. However, one or two alleles were shared between 'Valencí Blanc' and 'Valencí Tinto'. Therefore, the unknown parent from 'Valencí Blanc' could be the parent of 'Valencí Tinto'. The name of 'Valensi Chaselas' also appeared in the report of grapevines cultured in 1889, concretely as cultured in the Valencian location of Gandía.

‘Esclafagerres’ variety (Figure 2B), which name means that which bursts the jars, is also an ancient variety with white berries commonly grown on the Alicante and Valencia provinces. Some old references that mentioned the culture of this variety in the Alicante province are reported by several authors [65-67]. In DGAIC [52], the culture of ‘Esclafagerres’ appeared in the Alicante and in the Valencian locations of Albaida, Onteniente and Sagunto, where it was included among the varieties with white and also with black berries. This variety was usually mixed with other grapevine varieties like ‘Merseguera’ for wine production. The ‘Esclafagerres’ variety gives high yields (probably the meaning of the name is related to this) and has grapes with low sugar content despite it was commonly cultured under dry land.

In this work, we report the assays performed with both varieties in order to determine their genetic profiles and resume the strategies performed for virus sanitation and *in vitro* conservation.

Determining the SSR profiles

A total of 14 samples of ‘Valencí Blanc’ from different locations (Table 1) and two samples of ‘Esclafagerres’ from La Mata and Monforte del Cid were used for DNA extraction and SSR analysis. DNA was extracted from fully-expanded leaves using the commercial DNeasy Plant Mini Kit (Qiagen) according to the manufacturers’ instructions. DNA quality and quantity was assessed using gel electrophoresis and spectrophotometry. Fifteen SSR markers (VVS2, VVMD5, VVMD6, VVMD7, VVMD24, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, VrZAG64, VrZAG79, VrZAG83 and VMC1b11) were analyzed using two sets of multiplex PCR reactions. Each multiplex was carefully assembled according to the compatibility of the SSRs during PCR and the molecular size of their amplicons. The forward primer of the SSR markers was labeled with one of the four fluorescent dyes: carboxy fluorescein (FAM), carboxytetramethylrhodamine (TAMRA), hexachloro-6-carboxyfluorescein (HEX), or 6-carboxytetramethyl rhodamine (ROX) [61]. Multiplex PCR was carried out in a total volume of 11.00 μL, using 1.25 μL of commercial Master Mix PCR Multiplex (Takara Multiplex Hot Short PCR, Takara), 20-40 ng of genomic DNA, 0.1 μL of Takara Taq Hot Start and labeled multiplexed SSR primers (from 5.5 to 35.0 μmol). The amplification was performed in an ABI 9700 thermocycler, and the amplification conditions were 95 °C for 14 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 90 s, and 72 °C for 60 s, and a final extension of 72 °C for 30 min. Previous to PCR fragment size determination, the multiplex PCR product was previsualized using gel electrophoresis. The electrophoresis was carried out on an ABI 3100 platform (Applied Biosystems, Foster City, CA, USA). For PCR fragment size determinations, 0.13 μL of an internal size standard

(GeneSacn^{TM} 500 LIZ, Applied Biosystems) was mixed with 1.00 µL of PCR product and 10.87 µL of formamide. The mixture was heated at 94 °C for 3 min and then cooled in icy water. The size of the SSR fragments was determined with the software package GeneScan 3.7 (Applied Biosystems).

The SSR profiles of the analyzed accessions are shown at Table 1. Whereas similar SSR profiles were found for both accessions of 'Esclafacherre', some variability was found among 'Valencí Blanc' accessions. Among the 15 SSR analyzed, differences in the SSR VVMD32 were found: the accessions collected in the province of Alicante have two alleles in this loci VVMD32 (254; 270) whereas the rest of accessions from the provinces Badajoz, Cuenca, and Valencia have the allele 254 in homocigosity. We can consider that among the analyzed germplasm there are two variants of 'Valencí Blanc' as in other cases in which two plants showed identical SSR profiles for all the SSR markers studied except for one or two alleles. This could be attributable to slight clonal polymorphism [68]. They may have originated in a similar place and then spread to different areas. The accession of 'Valencí Blanc' in the VIVC database (accession number 22710) and that reported by Lacombe et al. [62] had the same profile for the comparable SSRs (including the VVMD32) to the accessions from Alicante. Similarly, accessions from Alicante showed also identical SSR profile to two accessions of 'Beba' from El Encín grapevine collection analyzed by Mena [64].

The comparison of the SSR profile of the 'Esclafagerres' accessions to SSR profiles in the VIVC database (including 3,265 accessions); those in the International Vitis database (including 3,430 accessions), as well as with those reported in several publications [62, 63, 69] did not match with any of the included varieties. No matches were neither found when the SSR profile of 'Esclafagerres' was blasted to the Italian Vitis database. Therefore, this profile should correspond with that of the 'Esclafagerres' variety which has not been reported before.

Virus analysis and sanitation

To analyze the putative virus infection in the original samples, the methodology described by López-Fabuel et al. [70] was used. Briefly, extracts were prepared from leaves 1/20 in phosphate buffered saline (PBS) buffer, pH 7.2, supplemented with 0.2% diethyldithiocarbamic acid (DIECA), and 2% polyvinylpyrrolidone-10 (PVP-10) in individual plastic bags with a heavy net (Plant Print Diagnostics). Total RNA was extracted from 200 µL of crude extract using an Ultraclean Plant RNA isolation kit (Mobio) following the manufacturer's instructions. The real-time multiplex RT-PCR (Reverse Transcription Polymerase Chain Reaction) was performed for the simultaneous detection of

ArMV, GFLV, GFkV, GLRaV-1, and GLRaV-3 using a StepOne Plus thermocycler (Applied Biosystems) and a reaction mixture containing 1x AgPath-ID One-step RT-PCR buffer (Ambion) and 1.5 x AgPath-ID One-step RT-PCR enzyme mix (Ambion); 5 µL of sample; 400 nM of GFLV, ArMV, GFkV, and GLRaV-1 primers; 800 nM of GLRaV-3 primers; and 200 nM of each probe. The amplification protocol consisted of an RT step at 45 ºC for 10 min and a denaturation step at 95 ºC for 10 min, followed by 45 cycles of amplification (95 ºC, 15 s; 50 ºC, 15 s; and 60 ºC, 60 s). As positive controls viral isolates maintained at the Instituto Valenciano de Investigaciones Agrarias (IVIA) were included. When amplification was observed for a specific virus, it was confirmed by real-time uniplex RT-PCR using the corresponding primers.

Meristem culture was used in a previous project (RTA2011-00067-C04) to obtain virus free plants of 'Esclafagerres'. In the context of the project CGL2015-70843-R, 'Valencí Blanc' (sample Vb-Pe0), which resulted infected with GFkV and GLRaV-3, was sanitized through both meristem culture and somatic embryogenesis. Meristems (n=35) from plants of 'Valencí Blanc' were extracted using a binocular lent and cultured *in vitro* on plates (90 x 15 mm) containing the medium MW, selected for 'Monastrell' micropropagation [71] supplemented with 6-benzylaminopurine (BAP) at 1.8 µM. Low light conditions were used for the two first weeks of culture. Only 54.3% of meristems grew after 20 days of culture (Figure 3A) and two develop into plants after transferring to tubes with MW, 70 days after (Figure 3B). Damage of meristem during extraction and/or the composition of the culture medium that could need to be enriched with other nutrients are putatively the causes of the low and slow regeneration of meristems. The analysis for virus presence of these two plants was carried out as described before and one of them (50%) resulted free for both viruses. Therefore, from this plant, clones were obtained for *in vitro* conservation. Despite the fact that meristem culture is an efficient technology for virus sanitation it is needed to obtain an adequate size of the meristem in order to avoid virus transmission allowing meristem development.

The other methodology used for virus sanitation was the induction of somatic embryos as reported in Peiró et al. [41]. Briefly, seeds of 'Valencí Blanc' were extracted from grapes, disinfected and cut previously to be cultured on the embryogenesis induction medium (EIM2) which contained TDZ (thidiazuron) at 0.9 µM. Thirteen per cent of explants responded after 60 days of culture on this medium (Figure 3C-D). Despite in grapevine a high percentage of somatic embryos are not able to develop into normal plants [72-74], in our work, germination of somatic embryos occurred directly in the induction medium and grew correctly (Figure 3E). Ten developed plants were analyzed for evaluating the success of virus cleaning. All plants regenerated from somatic embryos resulted virus

free for both GFkV and GLRaV-3 viruses. Therefore, 100% of sanitation was obtained. The result obtained for GFkV was expected because this virus is not seed-transmitted [75]. With respect to GLRaV-3, it is not clear if it is present in seeds [34, 75] but we have found this virus in some regenerated plants resulting from somatic embryos induced from seeds of other infected grapevines, which would indicate its presence in the seeds [54]. Induction of somatic embryos from stamens or pistils was also reported in grapevine to cure plants of GLRaV [76, 77]. We also analyzed the SSR profile of regenerated virus free plants in order to select those regenerated from mother tissues of the seeds, which will show the mother genotype. The 15 SSRs used for determining the SSR profile of 'Valencí Blanc' accessions were used. One of ten analyzed plants showed the same SSR profile as the mother plant, that is a 10% of regenerated plants were obtained from mother tissue and not from the embryo.

In vitro storage

Sanitized plants of 'Valencí Blanc' and 'Esclafagerres' are maintained in tubes with MW medium in an *in vitro* culture growth chamber under standard conditions (25 ºC ± 2 ºC; 16 h light). The MW medium is adequate to storage a broad spectrum of grapevine cultivars including 'Valencí Blanc' and 'Esclafagerres' [44]. Both cultivars grew less than 4 cm after 40 days of culture. We consider that this speed of growth is acceptable to maintain these cultivars under standard conditions with small number of subcultures. A reduction of sugar or the elimination of IBA in the culture medium is used for maintaining cultivars that grew faster [44]. The higher the number of subcultures, higher cost and higher possibility to make nomenclature errors [78].

Another strategy to germplasm *ex situ* storage using *in vitro* culture is cryopreservation. With this methodology, the metabolism is greatly reduced and few requirements are needed for the maintenance of tissue samples. Meristems of 'Esclafagerres' from micropropagated virus free plants maintained *in vitro* were used to initiate the cryopreservation assays using the methodology described in Gisbert et al. [79]. The first results indicated that 50 min of incubation in the plant vitrification solution 2 (PVS2) is adequate for recuperating cryopreserved meristems of 'Esclafagerres' (Figure 2). Recently, Pathirana et al [47] have reported a positive effect on grapevine regeneration when a pre-treatment with salicylic acid was performed previous cryopreservation. In both works [47, 79], the droplet vitrification protocol was used.

3. Conclusion

As a result of different actions performed in the context of the projects CGL2015-70843-R and RTA2011-00067-C04, a broad spectrum of grapevine varieties are being evaluated in order to determine the varietal identification and their variability, and also their capacity for *in vitro* culture, plant regeneration and germplasm storage. Different strategies for virus cleaning has been developed and applied to rescue virus free-plants. Among the analyzed materials, the historic varieties 'Valencí Blanc' and 'Esclafagerres' were sanitized and currently are maintained under *in vitro* culture conditions. Differences for the microsatellite VVMD32 were found among 'Valencí Blanc' accessions, clustering the accessions from Alicante and that of other origins. The SSR profile for the variety 'Esclafagerres' was firstly reported in the present work.

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Table 1. SSR profiles for 14 accessions of 'Valencí blanc' (Vb-Pe0, Vb-Pe1, Vb-Pe2, Vb-Pe3, Vb-Pe4, Vb-Pe5, Vb-Pe10, Vb-Be1, Vb-Al1, Vb-FF1, Vb-FA1, Vb-On1, Vb-Ba1, Vb-Cu1) and two of 'Esclafagerres' (Es-Ma1, Es-Mo1) varieties. Allele sizes are expressed as base pairs.

Code	Origin	VVMD27		VVMD5		VVS2		VrZAG83		VrZAG79		VrZAG62		VrZAG64		VVMD7		VVMD24		VVMD32		VVMD25		VMC1b11		VVMD28		VVMD6		VVMD21	
		A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
Vb-Pe0	Penàguila (Alicante)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	270	255	255	185	189	243	257	209	211	248	254
Vb-Pe1	Penàguila (Alicante)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	270	255	255	185	189	243	257	209	211	248	254
Vb-Pe2	Penàguila (Alicante)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	270	255	255	185	189	243	257	209	211	248	254
Vb-Pe3	Penàguila (Alicante)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	270	255	255	185	189	243	257	209	211	248	254
Vb-Pe4	Penàguila (Alicante)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	270	255	255	185	189	243	257	209	211	248	254
Vb-Pe5	Penàguila (Alicante)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	270	255	255	185	189	243	257	209	211	248	254
Vb-Pe10	Penàguila (Alicante)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	270	255	255	185	189	243	257	209	211	248	254
Vb-Be1	Benirarres (Alicante)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	270	255	255	185	189	243	257	209	211	248	254
Vb-Al1	Alicante	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	270	255	255	185	189	243	257	209	211	248	254
Vb-FF1	La Font de la Figuera (Valencia)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	*254*	255	255	185	189	243	257	209	211	248	254
Vb-FA1	Fontanars dels Alforins (Valencia)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	*254*	255	255	185	189	243	257	209	211	248	254
Vb-On1	Ontinyent (Valencia)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	*254*	255	255	185	189	243	257	209	211	248	254
Vb-Ba1	Fuente del Maestre (Badajoz)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	*254*	255	255	185	189	243	257	209	211	248	254
Vb-Cu1	Campillo de Altobuey (Cuenca)	179	187	233	237	136	144	197	197	242	246	189	205	134	158	241	247	206	208	254	*254*	255	255	185	189	243	257	209	211	248	254
Es-Ma1	La Mata (Alicante)	187	191	233	237	146	152	193	197	242	246	189	197	134	136	237	247	206	208	254	270	241	255	171	189	233	247	209	211	248	248
Es-Mo1	Monforte del Cid (Alicante)	187	191	233	237	146	152	193	197	242	246	189	197	134	136	237	247	206	208	254	270	241	255	171	189	233	247	209	211	248	248

Each code corresponds to a Valencí Blanc (Vb) or Esclafacherre (Es) accession collected in a location. Different numbers were assigned for different accessions collected at the same location.

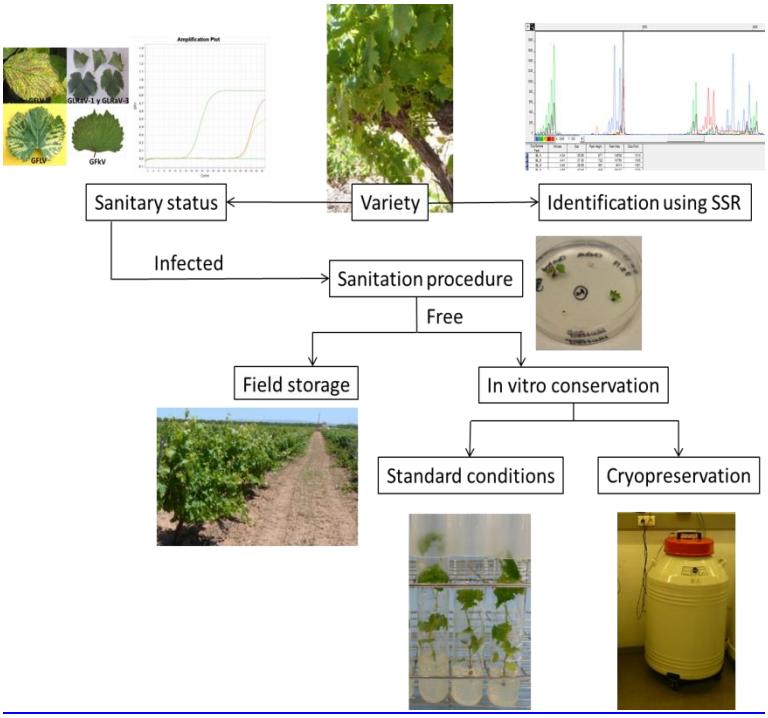

Figure 1. Scheme including the main steps required for germplasm storage.

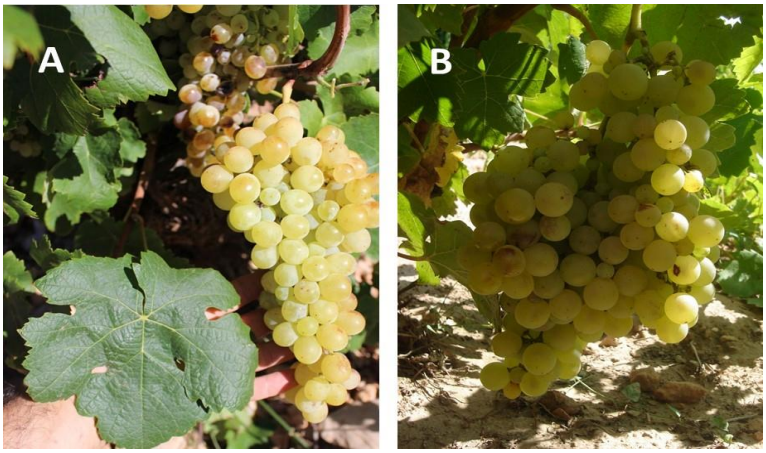

Figure 2. Grapes of ‘Valencí blanc’ (A) and ‘Esclafagerres’ (B) varieties.

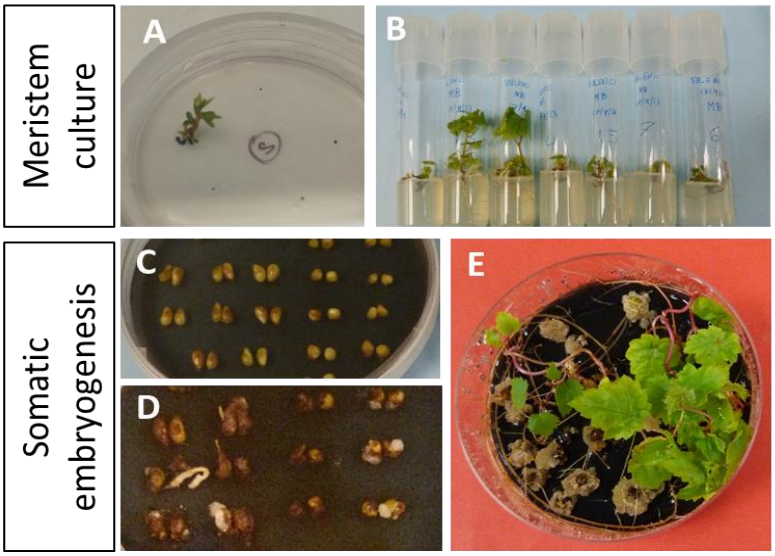

Figure 3. Strategies for virus sanitation of 'Valencí Blanc' (accession Vb-Pe0). (A) Meristems cultured on MW supplemented with 6-benzylaminopurine (BAP) at 1.8 µM and without indole butyric acid (IBA) after 20 days of culture. (B) Plants from meristems cultured in MW medium after 90 days of culture. (C) Cut seeds cultured in EIM2 medium at day 0. (D) Seeds with some somatic embryos after 45 days of culture. E. Plants regenerated from somatic embryos.

Capítulo 5

Attempts at grapevine (*Vitis vinifera* L.) breeding through genetic transformation: The main limiting factors

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Attempts at grapevine (*Vitis vinifera* L.) breeding through genetic transformation: The main limiting factors

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Summary

Breeding through genetic transformation offers the possibility to add or modify single traits in cultivars without changing desirable characteristics. In grapevine this technology has been scarcely used, taking into account the economic importance of this crop. Up to now, the breeding of grapevine through genetic transformation has been mainly focused on biotic stress resistance, mainly to fungi and viruses. Among the factors that can explain the limited reports of success are the difficulty in regenerating transgenic plants, the availability of only a few characterized genes, and/or the quantitative character of the trait. Another influencing factor is the negative perception of consumers, mainly in Europe. In this review, we discuss the methodology and factors that have limited the success of grapevine transformation, as well as outlining the attempts at breeding grapevine through genetic transformation reported so far. It is expected that the use of transformation, a powerful tool for breeding plants, will increase in grapevine in the coming years as a consequence of the growing knowledge of the function and regulation of grapevine genes and promotors, and of technologies for gene editing.

Introduction

Grapevine (*Vitis vinifera* L.) production had an international value of 44 billion dollars in 2013 (FAOStat 2013) and the global production of grapes was 27.5 million tones. Grapevine was domesticated more than 5000 years ago (YAMAMOTO *et al*. 2000) and it is considered nowadays the world's most widely-grown fruit crop (MUKHERJEE *et al*. 2010). Although the genus *Vitis*

includes ca. 60 inter-fertile species, *V. vinifera* is the one used most in the global wine industry. *V. lambrusca*, native to North America, also contributes to the development of hybrids for wine and juice production, in certain areas where the climatic conditions may limit *V. vinifera* production (LEE *et al*. 2006). Other species of this genus are employed as grapevine rootstocks due to their resistance to pests (Phylloxera, nematodes), drought tolerance, salt tolerance or tolerance of high pH (KELLER 2010). Although there are almost 10000 cultivars of grapevine (ROBINSON *et al*. 2012), only a few - including 'Cabernet Sauvignon', 'Merlot', 'Airén', 'Tempranillo', 'Chardonnay', 'Syrah', 'Garnacha Tinta', 'Sauvignon Blanc', 'Trebbiano Toscano', and 'Pinot Noir' - are grown widely (ANDERSON and NELGEN 2011, www.research.wineaustralia.com).

The breeding of scion cultivars has been focused mainly on obtaining resistance to different pathogens. Among the most devastating fungal diseases are powdery mildew, anthracnose, downy mildew, and gray mold rot, caused by *Erysiphe necator*, *Elisinoe ampelina*, *Plasmopara viticola*, and *Botyris cinerea*, respectively (DE FRANCESCO 2008, WILCOX 2011). Other important infections are caused by bacteria like *Xylophilus ampelinus*, which can lead to a serious reduction in grapevine health and major harvest losses (SERFONTEIN *et al*. 1997), *Xylella fastidiosa*, which causes Pierce's disease, which can kill the vine in one or two years (HILL and PURCELL 1995, JANSE and OBRADOVIC 2010), and *Agrobacterium tumefaciens* biovar 3 (*Agrobacterium vitis*), which causes crown gall disease and induces tumors at wounds on the trunks and canes of the grapevine that can necrotize the plant (BURR *et al*. 1998, RIDÉ *et al*. 2000). Viruses cause other diseases that greatly affect grapevines, especially the Grapevine FanLeaf Virus (GFLV), belonging to the genus *Nepovirus*, which causes the grapevine fanleaf disease. Other breeding goals for grapevine are related to abiotic stress tolerance and quality improvement (VIVIER and PRETORIUS 2002, GRAY *et al*. 2014).

Genes conferring resistance to several pathogens have been introgressed into *V. vinifera* cultivars (ALLEWELDT 1990; MULLINS *et al*. 2004). However, the high level of heterozygosity and the long generation cycle in grapevine make breeding by conventional methods difficult (NAKANO *et al*. 1994, FRANKS *et al*. 1998). Therefore, breeding through genetic transformation has been another approach used to incorporate desirable genes into grapevine. This technique offers the possibility of adding single traits to cultivars without, in theory, changing desirable characteristics (GRAY *et al*. 2005). The sequencing of the *V. vinifera* genome (JAILLON *et al*. 2007, VELASCO *et al*. 2007, ADAM-BLONDON *et al*. 2011) - which contains over 30,400 genes (PERTEA and SALZBERG 2010) - has yielded the possibility of obtaining more cisgenic plants; that is, those containing genes or

regulatory sequences in a sense orientation, which have been isolated from a crossable donor plant. In the work of SCHOUTEN *et al*. (2006), FAN *et al*. (2008), DHEKNEY *et al*. (2011), DABAUZA *et al*. (2015), DAI *et al.* (2015), and DALLA-COSTA *et al*. (2015a), *V. vinifera* genes were introduced mainly by genetic transformation mediated by *Agrobacterium tumefaciens* (updated scientific name *Rhizobium radiobacter*). The availability of genes and emergent technologies, like the CRISPR/Cas system for genome modification (WANG *et al.* 2016) should increase the breeding attempts in grapevine.

Here, we provide an overview of the published work in which attempts at the stable transformation of *V. vinifera* were made, discussing the methodology and factors that have limited the success of grapevine transformation.

Discussion

Despite the fact that *Vitis* sp. is considered to be a natural host for *A. tumefaciens* (BORNHOFF *et al*. 2005), T-DNA transfer and its integration into the plant genome by *Agrobacterium* sp. as well as by biolistic methods have been used to incorporate specific genes. To the best of our knowledge, the first attempt to obtain stable transgenic plants in grapevine was reported in 1989 by BARIBAULT *et al*., who used *A. tumefaciens* infection to introduce the neomycin phosphotransferase gene (*nptII*), which confers resistance to kanamycin (Kan), into the cultivar 'Cabernet Sauvignon'. These and other early studies (BARIBAULT *et al*. 1990, MULLINS *et al*. 1990, GUELLEC *et al*. 1990) met with limited success. With respect to biolistics, HÉBERT *et al*. (1993) reported the first transformation in 'Chancellor', a *Vitis* complex interspecific hybrid, and SCORZA *et al*. (1995) were the first group to achieve transformation by a biolistic approach in seedless table grapes. Nowadays, biolistics is the method of choice for studying transient expression for functional analysis. Recent reviews concerning transient expression were provided by VIDAL *et al*. (2010) and JELLY *et al*. (2014). Regarding the use of genetic transformation for the breeding of grapevine plants, *Agrobacterium*-mediated transformation has been used mainly (Table 1). The combination of both methodologies (biolistics previous to *Agrobacterium* infection) was also tested by SCORZA *et al*. (1995 & 1996). One of the advantages of *Agrobacterium*-mediated transformation, with respect to biolistic methods, is the low gene copy number that is obtained in plants regenerated with the former methodology (LI *et al*. 2006, DUTT *et al*. 2008).

Despite the great interest in this technology, it has not been used extensively because of the failure of genetic transformation and/or of the recovery of transgenic plants (SCORZA *et al.* 1996, NOOKARAJU and AGRAWAL 2012).

Limiting factors: the genotype

Independent of the methodology of gene transfer, the most influential factor regarding the success of transformation is the genotype. Before an attempt to generate transgenic plants is made, an efficient protocol of adventitious regeneration is needed; this will be greatly influenced by the genotype and culture conditions (explant, culture media, etc.). Interspecific and intraspecific variability for regeneration ability were commonly found. Different QTLs have been related to regeneration in different species (PRIYONO *et al.* 2010, TRUJILLO-MOYA *et al.* 2011, ZHENZHEN *et al*. 2015), which manifests the implication of several genes in the regeneration process. On the other hand, each genotype manifests a specific sensitivity to the *Agrobacterium* strain, as well as to the selective agents added to the medium to impede regeneration from non-transgenic cells and to the antibiotics applied to eliminate *Agrobacterium* after transformation (ZHOU *et al.* 2014). Differing, genotype-dependent toxicity may also be manifested in biolistic assays according to the type of particle that carries the DNA (FRANKS *et al*. 1998, VIDAL et al. 2003).

In *Agrobacterium*-mediated transformation, success will be influenced also by the ability of the cells of each genotype to be transformed, the concentration of bacteria, the time of co-culture, and even the genetic constructions used. In biolistic transformation, the size of particles coated with DNA, the helium pressure, the gap distance, the vacuum, and the distance from the carrier to the sample also may be factors that will influence the transgenic efficiency. Both gold (SCORZA *et al*. 1995 & 1996, FRANKS *et al.* 1998, VIDAL *et al*. 2003 & 2006) and tungsten (HÉBERT *et al*. 1993, VIDAL *et al*. 2003) particles, of 0.6, 0.75, 1, and 1.6 microns in size, have been used to carry the DNA in grapevine.

Adventitious regeneration

Adventitious regeneration may occur via organogenesis or embryogenesis. In *Vitis* sp. adventitious regeneration is mainly achieved through somatic embryogenesis and much research has been carried out using, as starting explants: leaves (MARTINELLI *et al*. 1993, NAKANO *et al*. 1994, SCORZA *et al*. 1995 & 1996, DAS *et al*. 2002, BORNHOFF *et al*. 2005, LI *et al*. 2006, DUTT *et al*. 2008, NIRALA *et al*. 2010, DHEKNEY *et al*. 2011, NOOKARAJU and AGRAWAL 2012, LI *et al.* 2015),

anthers (FRANKS *et al*. 1998, IOCCO et *al*. 2001, NAKAJIMA and MATSUDA 2003, VIDAL *et al*. 2003 & 2006, PERRIN *et al*. 2004, AGÜERO *et al*. 2005, GAMBINO *et al*. 2005, FAN *et al*. 2008, LÓPEZ-PÉREZ *et al*. 2008, ROSENFIELD *et al*. 2010, WANG *et al*. 2005, DAI *et al.* 2015), ovaries (YAMAMOTO *et al*. 2000, VIDAL *et al*. 2003 & 2006, GAMBINO *et al*. 2005, ROSENFIELD *et al*. 2010, DAI *et al*. 2015), whole flowers (DAI *et al.* 2015), mature seeds (PEIRÓ *et al*. 2015), stigmas and styles (MORGANA *et al.* 2004, CARIMI *et al.* 2005), petioles (ROBACKER 1993), tendrils (SALUNKHE *et al.* 1997), nodal sections (MAILLOT *et al.* 2006), and protoplasts (REUSTLE *et al*. 1995, ZHU *et al*. 1997, XU *et al*. 2007). Although regeneration has been obtained in several cultivars, the germination of aberrant embryos that may limit regeneration or decrease the real percentage of regenerated grapevine plants is a common occurrence. Embryos without cotyledons, with different numbers of cotyledons (mono-, di-, and poly-cotyledonary), or with fused cotyledons, and trumpet-shaped or cauliflower-like cotyledons were described in different works (GOEBEL-TOURAND *et al*. 1993; MARTINELLI *et al*. 2001; BORNHOFF *et al.* 2005, LI *et al*. 2006; LÓPEZ-PÉREZ *et al*. 2006; BHARATHY and AGRAWAL 2008; MARTINELLI and GRIBAUDO 2009 or PEIRÓ *et al.* 2015). The majority of these abnormal embryos do not develop into normal plants. For grapevine transformation three types of embryogenic tissue are commonly used: somatic embryos from leaves (SCORZA *et al*. 1995 & 1996, LI *et al*. 2006, DUTT *et al*. 2008, DHEKNEY *et al.* 2011, NOOKARAJU and AGRAWAL 2012, LI *et al*. 2015), anthers (FRANKS *et al*. 1998), or ovules (YAMAMOTO *et al.* 2000); embryogenic calli from leaves (NAKANO *et al*. 1994, NIRALA *et al*. 2010), anthers (FRANKS *et al*. 1998, IOCCO *et al*. 2001, AGÜERO *et al*. 2005, GAMBINO *et al*. 2005, FAN *et al*. 2008, LÓPEZ-PÉREZ *et al*. 2008, DAI *et al*. 2015), ovaries (GAMBINO *et al*. 2005, DAI *et al*. 2015), or whole flowers (DAI *et al*. 2015); and cell suspensions from anthers (FRANKS *et al*. 1998, VIDAL *et al.* 2003 and 2006, WANG *et al*. 2005, ROSENFIELD *et al*. 2010) or ovaries (VIDAL *et al*. 2003 & 2006, ROSENFIELD *et al*. 2010). Leaf disks, microshoots, or meristematic cell clusters were also used by BORNHOFF *et al*. (2005), GAGO *et al*. (2011), and MEZZETTI *et al*. (2002a), respectively.

About half of the reports in Table 1 used ‘Chardonnay’ and ‘Thompson Seedless’ for grape transformation and great variability was reported with respect to the number of plants regenerated in selective conditions. In IOCCO *et al*. (2001), the effect of genotype is clear. With similar transformation conditions, the number of plants regenerating under selective conditions (Kan applied 3 weeks after co-culture) greatly differed among cultivars: 161 plants of ‘Shiraz’, 136 ‘Cabernet Sauvignon’, 57 ‘Chenin Blanc’, 52 ‘Chardonnay’, 23 ‘Sauvignon Blanc’, 19 ‘Riesling’, and nine ‘Muscat Gordo Blanco’ were obtained, but no ‘Semillon’ or ‘Pinot Noir’.

Effects of the Agrobacterium strain and selective agent on regeneration

In different studies, it is reported that the competence of *Agrobacterium* mediated transformation is cultivar dependent (IOCCO *et al*. 2001, DUTT *et al*. 2011). In addition, after *Agrobacterium* inoculation, necrosis and tissue browning - as a result of an oxidative burst caused by reactive oxygen species - may appear in some genotypes (PERL *et al*. 1996, GUSTAVO *et al*. 1998, LI *et al.* 2006; ZHOU *et al*. 2014), affecting regeneration. These facts, associated with the sensitivity to the selective agents (which depend on the gene inserted for selection), together with the putative sensitivity to the antibiotics used for elimination of *Agrobacterium* (that will differ depending on the virulence of the bacteria), explain the difficulty in obtaining efficient protocols for grapevine transformation.

Among the disarmed *A. tumefaciens* strains LBA4404 (HOEKMA *et al.* 1983), GV2206 (RYDER *et al.* 1985), EHA101 and EHA105 (HOOD *et al.* 1993), GV3101 (HOLSTERS *et al.* 1980), and AGL1 (LAZO *et al.* 1991) (all derive from C58, with the exception of LBA4404 - that derives from Ach5), EHA105 is the one employed most in grapevine transformation (SCORZA *et al*. 1996, FRANKS *et al*. 1998, IOCCO *et al*. 2001, WANG *et al*. 2005, DHEKNEY *et al*. 2007 & 2011, DUTT *et al*. 2008, DABAUZA *et al*. 2015, LI *et al.* 2015). Despite the fact that regeneration was obtained by NAKANO *et al*. (1994) and FRANKS *et al*. (2006) using *Agrobacterium rhizogenes*, no more reports were found in grapevine.

In order to restrict regeneration to cells which have incorporated the transgene, the *nptII* gene (Table 1) - that confers resistance to kanamycin (Kan) and other antibiotics like paramomycin, neomycin, and G418 - has been the one used most commonly in grapevine transformation (NAKANO *et al*. 1994, SCORZA *et al*. 1995 & 1996, FRANKS *et al*. 1998, YAMAMOTO *et al*. 2000, IOCCO *et al*. 2001, VIDAL et al. 2003 & 2006, AGÜERO *et al*. 2005, BONHOFF *et al.* 2005, GAMBINO *et al*. 2005, WANG *et al.* 2005, LI *et al*. 2006, DUTT *et al*. 2008, LÓPEZ-PÉREZ *et al*. 2008, JIN *et al*. 2009, DHEKNEY *et al*. 2011, GAGO *et al*. 2011, DABAUZA *et al*. 2015, LI *et al.* 2015). However, high sensitivity of grapevine tissues to Kan was reported in GRAY and MEREDITH (1992) and different authors mention that it is really difficult in grapevine to balance the concentration of Kan that is adequate for selection with that which allows development of embryos and shoots (GRAY and MEREDITH 1992, TORREGROSA *et al*. 2000, SAPORTA *et al.* 2014). This explains the great differences with respect to the Kan concentration used for selection and the time of application of the selective agent (Table 1). In addition to the genotype, the kind of explant used (callus, suspension cultures…) for transformation will lead to differences in sensitivity (ZHOU *et al*. 2014). Kanamycin is used in a range from 10 to 100 $mg.L^{-1}$ (Table 1). Regarding the application of the selective agent,

FRANKS *et al*. (1998) compared three strategies, achieving better results when they applied low selection (2 mg.L^{-1} Kan) at the beginning, moderate selection (50 mg.L^{-1} Kan) three weeks after co-cultivation, and 100 mg.L^{-1} Kan thereafter. Good regeneration was also obtained by IOCCO *et al*. (2001), who added 100 mg.L^{-1} Kan after three weeks of co-culture. Although they found a high number of escapes, some transgenics were selected in all the cultivars showing regeneration. In WANG *et al.* (2005), Kan and paromomycin were compared as selective agents for 'Red Globe', with better results for transformation efficiency and embryo development being obtained with the latter at 20 mg.L^{-1}. However, the use of this antibiotic is not common in transgenic work.

The *hptI* gene, that encodes hygromycin (Hyg) phosphotransferase I, is the second selective gene used, to select transformed grapevine cells in Hyg-containing media (FRANKS *et al*. 1998, FAN *et al.* 2008, NIRALA *et al*. 2010, NOOKARAJU and AGRAWAL 2012, DAI *et al.* 2015). The Hyg concentrations employed ranged from 3 to 25 mg.L^{-1} (FRANKS *et al*. 1998, FAN *et al*. 2008, NIRALA *et al*. 2010, NOOKAJARU and AGRAWAL 2012, DAI *et al*. 2015). Whereas FRANKS *et al.* (1998) obtained a good selection efficiency using 25 mg.L^{-1} Hyg (11 of 12 regenerated plants of cv. 'Sultana' were confirmed as transgenic), FAN *et al.* (2008) found toxicity during direct selection with 12 mg L^{-1} Hyg in 'Thompson Seedless'. A stepwise selection with 3, 6, 9, and finally 12 mg L^{-1} gave a high transformation efficiency (72%). In NIRALA *et al*. (2010) a stepwise selection was also performed, for the cultivar 'Pusa Seedless'; however, in this work the first selection was made with 10 mg.L^{-1} Hyg. This concentration was also used for selection in 'Chardonnay' by DAI *et al*. (2015), who recovered normal and abnormal embryos (deformed leaves, vitrification, no development of roots). In SAPORTA *et al.* (2014) a comparison of Kan and Hyg was performed for cellular suspensions of cultivar 'Albariño', yielding an optimal selection pressure of 20-40 mg.L^{-1} and 5-10 mg.L^{-1} for the former and the latter antibiotic, respectively.

The genes *bar* (PERL *et al.* 1996) and *pmI* (REUSTLE *et al*. 2003, KIEFFER *et al*. 2004, JARDAK-JAMOUSSI *et al*. 2008) - that encode, respectively, a phosphinothricin acetyl transferase and a phosphomannose isomerase - have also been used as selective agents in grapevine transformation, with success.

Even when using similar selective conditions (100 mg.L^{-1} Kan, 3 weeks after co-culture) and explant type (embryogenic cultures from immature anthers), different regeneration was obtained for a specific cultivar. For instance, in 'Shiraz', IOCCO *et al.* (2001) obtained 161 plants whereas only eight were reported in TORREGROSA *et al.* (2002). In the former study 28% integration was found, with 25% in the latter. In the cultivar 'Chardonnay', TORREGROSA *et al*. (2002) could not regenerate plants;

however, IOCCO *et al*. (2001) obtained 52 plants (13% confirmed as transgenic). Probably, these great differences are due to other factors that influence the protocol.

In some studies confirmation of integration and/or expression was not performed, the authors assuming that the plants regenerated under selective conditions were transgenic (IOCCO *et al*. 2001, LI *et al*. 2006, WANG *et al*. 2005, BORNHOFF *et al*. 2005). From the results in Tab. 1 we can conclude that, despite the fact that in some cases 100% of the regenerated plants were confirmed as transgenic, in the majority of the studies escapes were regenerated.

The European Food Safety Authority (EFSA 2009) recognizes *nptII* as a safety gene; however, different strategies have been developed and are available to recover *nptII*-free plants after selection. In grapevine a co-transformation system was reported by DUTT *et al*. (2008), who were trying to produce transgenic grapevines free of marker genes. They used an *Agrobacterium* strain which contained a binary plasmid with an *egfp* gene of interest for positive selection and, for negative selection, the cytosine deaminase (*codA*) gene, the two genes linked by a bi-directional dual promoter complex. DALLA-COSTA *et al*. (2009 & 2010) employed the XVE-Cre/LoxP system to induce removal of the *nptII* gene, induced by 17-β-estradiol. Calli, leaves, and roots from the Italian cultivar 'Brachetto' were used and transgenic plants without the *nptII* gene were obtained, suggesting that the use of XVE-Cre/LoxP could be a good method for elimination of selectable gene markers.

Attempts at grapevine (Vitis vinifera L.) breeding through genetic transformation

Breeding for resistance to fungi and bacteria

As a consequence of pathogen attack, a number of pathogenesis-related (PR) proteins are produced in grapevine (JACOBS *et al*. 1999); among them the glucanases and chitinases are the most common. Besides PR proteins, the accumulation of phytoalexins - such as stilbenes - is the other major defense mechanism frequently observed in grapevine (FERREIRA *et al*. 2004). These kinds of antifungal related genes have been the ones used most commonly for grapevine breeding through genetic transformation (Table 2). For instance, the rice chitinases (*RCC2* and *Chil1*) were introduced by YAMAMOTO *et al*. (2000) and NIRALA *et al*. (2010) into 'Neo Muscat' and 'Pusa Seedless', respectively. In these works, the transformants had higher levels of chitinase activity and tended to have smaller lesions when they were affected by anthracnose and powdery mildew, with respect to the control plants. KIKKERT *et al.* (2000) also transformed 'Merlot' and 'Chardonnay' with an endochitinase gene from *Trichoderma harzianum* (*ThEn-42*), obtaining similar results: the

transformants had 10 to 100-fold higher chitinase activity relative to the controls but showed low levels of resistance to powdery mildew and a reduced incidence and severity of symptoms for Botrytis bunch rot, in both greenhouse and field evaluations (KIKKERT *et al*. 2009). Other biocontrol agents derived from *Trichoderma* spp. - like two endochitinase (*ech42* and *ech33*) genes and one *N*-acetyl-b-D-hexosaminidase (*nag70*) gene - were introduced into 'Thompson Seedless' by RUBIO *et al*. (2015), who obtained several lines with consistent resistance. Recently, DAI *et al.* (2016) used a *Vitis pseudoreticulata* PR gene (*VpPR4-1*) to transform the cv. 'Red Globe'. Six plants inoculated with powdery mildew showed resistance. The use of other chitinases, in combination with ribosome inactivation proteins (HARTS *et al*. 2000a; BORNHOFF *et al*. 2005) or beta 1-3 glucanases (HARST *et al*. 2000a; NOOKARAJU and AGRAWAL 2012), has also yielded results similar to those of the above mentioned works. Other plant PR proteins are the thaumatin-like proteins (TLPs), which are grouped into the PR-5 family. A *V. vinifera* gene (*Vvtl-1*) encoding a TLP was introduced into 'Thompson Seedless' and enhanced resistance to foliar fungal diseases and lowered the incidence of sour rot in berries (DHEKNEY *et al*. 2011). Also, genes encoding stilbene synthase - a key enzyme that produces trans-resveratrol, the major phytoalexin in grape - were introduced into 'Chardonnay' (DAI *et al*. 2015), 'Sugraone' (DABAUZA *et al*. 2015), and 'Thompson Seedless' (FAN *et al*. 2008). Reduced numbers of powdery mildew conidia and smaller lesions after infection with *B. cinerea* were reported in the first and second studies cited.

Other strategies to achieve resistance to fungi were the insertion of a polygalacturonase inhibiting protein (AGÜERO *et al.* 2005) and the introduction of antimicrobial genes (lytic peptides) like Shiva-1, *mag2*, *MSI99*, and *PGL* (SCORZA *et al.* 1996, VIDAL *et al*. 2003 & 2006, ROSENFIELD *et al*. 2010) (Table 2). Whereas the low transformation efficiency impeded the evaluation of resistance by SCORZA *et al*. (1996), a delay in the *A. vitis* infection or in the expansion of lesions in transgenic lines, with respect to the control, was reported by KIKKERT *et al*. (2009) in plants with *mag-2* and *MSI-99* genes. More recently, DANDEKAR *et al.* (2012) introduced a PGIP signal peptide with a cecropin derived lytic domain and LI *et al*. (2015) introduced the gene *LIMA-A* (that also encodes a lytic peptide derivative of *MsrA1*), in order to confront Pierce's disease. Although the plants showed resistance in the greenhouse, no durable resistance was obtained in the field - where all plants died before the seventh year of cultivation.

Transgenic grapevines for resistance to viruses and other pathogens

The first authors to obtain virus resistant grape plants through genetic transformation, like in other species, used pathogen-derived resistance: concretely, the insertion of virus coat proteins (CP) (Table

3). MAURO *et al*. (1995) and SCORZA *et al*. (1996) reported the transformation of cultivars 'Chardonnay' and 'Thompson Seedless' with a CP of GFLV and the Tomato Ringspot Virus CP (TomRSV-CP). A similar strategy was used in different studies which attempted to achieve GFLV resistance (GÖLLES *et al*. 1997; TSVETKOV *et al*. 2000; GUTORANOV *et al*. 2001 and GAMBINO *et al*. 2005) or resistance to ArMV and Grapevine viruses A and B (GVA, GVB) (GÖLLES *et al*. 1998). Movement virus proteins were also used by MARTINELLI *et al*. (1998) to achieve resistance to GVA and GVB. Resistance was not reported in these works. More recently, the RNA interference strategy was utilized for stable grapevine transformation, using inverted repeats (JARDAK-JAMOUSSI *et al*. 2008). In this case, a low number of transgenic lines of grapevine were obtained and evaluation of GFLV resistance was not reported.

Important pests of grapevine are phylloxera (*Daktulosphaira vitifoliae*) and root knot nematodes (RKN). Despite the problems caused by the former, that are solved by the use of resistant rootstocks, FRANKS *et al*. (2006) introduced, by genetic transformation, three sequences of Sorghum in order to produce a cyanogenic glycoside that is involved in plant defense mechanisms. However, these transformed plants did not show evidence of greater protection - probably because, after infestation, the accumulation of the metabolite was low. With respect to RKN, yield problems have increased since the withdrawal of methyl bromide. These pests, in addition to reducing yield because their galls limit nutrient acquisition, are virus transmitters. For instance, the dagger nematode (*Xiphinema index*) transmits GFLV, one of the most severe virus diseases of grapevines worldwide. In 'Chardonnay', YANG *et al*. (2013) introduced two hairpin-based silencing constructs, containing two stem sequences of the *16D10* gene, and transformed hairy roots to test their small interfering RNA (siRNA) production and efficacy of suppression of nematode infection, with promising results. They obtained four lines and better nematode resistance (fewer eggs per root) was observed, with respect to the control.

Cold tolerance, yield, and grape quality

Other reported attempts at enhancing cold tolerance and grape quality in grapevine through genetic transformation are shown in Table 4.

Plant stress responses take place through complex and interacting pathways, which indicate the difficulty for breeding with both traditional and biotechnological techniques. However, in grapevine different attempts have been made to achieve cold tolerance. The expression of an Fe-superoxide dismutase or the *VvAdh2* gene of *V. vinifera* - that encodes an alcohol dehydrogenase - was reported

by ROJAS *et al*. (1997) and TESNIERE *et al*. (2006), respectively. In the first of these works a lower sucrose content, a higher degree of polymerization of proanthocyanidins, and an increase in volatile compounds, especially for carotenoid - and shikimate-derived volatiles, were obtained in transgenic plants. On the other hand, JIN *et al*. (2009) and GUTORANOV *et al*. (2001), respectively, transformed 'Centennial Seedless' and 'Rusalka' grapevines with genes encoding different antifreeze proteins. They analyzed amino acids and found higher levels of alanine (approximately 14% higher than in the control plants) in a transformed grapevine, but cold tolerance was not evaluated in these studies.

Breeding for abiotic and biotic stress tolerance is breeding for yield. However, the modification of genes involved in other processes - such as root development, flower production, and fruit set - may also be a strategy to increase yield. In grapevine, an increase in the number of flowers and berries was reported by MEZZETTI *et al*. (2002a) and CONSTANTINI *et al*. (2007) in 'Thompson Seedless' and 'Silcora' transformed with *DefH9-iaaM*. Whereas in the first study the number of flowers was almost doubled in transgenic plants, with respect to the controls, in the second flower number increased only slightly. An increase in productivity (number of flowers or fruit size) and parthenocarpy has been obtained in transgenic eggplant (DONZELLA *et al*. 2000) or strawberry (MEZZETTI *et al*. 2002b) plants expressing this gene whereas similar productivity was obtained in transgenic tomato and the respective controls by FICCADENTI *et al*. (1999).

With respect to breeding for quality, this can be achieved indirectly; for instance, when obtaining resistance to a fungus. However, breeding specifically for quality is difficult because this is a complex trait that includes external and internal parameters that are also influenced by the climatic conditions and cultural practices. Therefore, more knowledge is needed to modify grapevine quality with precision by genetic transformation. For instance, MADS-box genes encode transcription factors that are associated with numerous developmental processes - including induction of flowering, specification of inflorescence and flower meristems, establishment of flower organ identity, and regulation of fruit, seed, and embryo development. Recently, GRIMPLET *et al*. (2016) identified a total of 90 MADS-box genes in the grapevine reference genome. An important berry quality trait is the sugar composition (glucose and fructose in the vacuole of flesh cells). Sugar signaling in grape is concerned mainly with the regulation of anthocyanin biosynthesis and sugar transport, but also with other major processes such as cell growth (DAVIES *et al*. 2012; LECOURIEUX, 2014). Volatile and non-volatile terpenoids - that greatly influence the varietal character of grapes and subsequently of wine (LUND and BOHLMANN 2006) - are other candidates for quality modification. Polyphenolics

(flavonoids and non-flavonoids) also contribute to the taste, astringency, color, and mouthfeel of wine (LUND and BOHLMANN 2006). Successfully, THOMAS and SCOTT (2001) transformed a seedless cultivar with a UDP: flavonoid 3-*O*-glucosyltransferase (UFGT), to control the color development of grape berries.

Conclusion

Up to now, the breeding of grapevine through genetic transformation has been mainly focused on biotic stress resistance, mainly to fungi and viruses. Other attempts have been related to cold tolerance and modification of berry color. The majority of these studies met with little success due to the difficulty in regenerating plants, the availability of few characterized genes, and/or the quantitative character of the trait. Also, in the majority of these studies, a test of the theoretical resistance obtained in transformed plants was not reported. Comparing the efficiency of genetic transformation among the published studies is really difficult and imprecise because different cultivars, as well as distinct protocols for transformation and regeneration in selective conditions, were used. However, based on the reported work, we can conclude that *Agrobacterium*-mediated transformation is the method of choice for stable transformation of grapevine and that most of the research performed to date has concentrated on 'Chardonnay' and 'Thompson Seedless'. Among the selective agents, *nptII* was the preferred gene for selection despite the sensitivity to kanamycin of some cultivars, for which the concentrations and time of application need to be adjusted to achieve regeneration. When using kanamycin and hygromycin as selective agents, a stepwise procedure seems the most appropriate to obtain regeneration of transgenics, although the regeneration of escapes together with plants that have integrated the transgene is common.

Currently, a great increment in grapevine breeding efforts is expected due to the greatly increased knowledge of gene function and regulation, and of the new promoters and technologies for gene editing, transfer, and selection (CUTT *et al*. 2014; BORTESI and FISCHER 2015). Therefore, new or modified protocols that facilitate the recovery of a large number of plants in a broad number of grapevine cultivars and rootstocks, in order to select those with a single integration and correct expression, are required.

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Table 1. Reports of stable transformation in grapevine: methodology and regeneration of transgenic plants

Cultivar	Method	Selection	Number of regenerated plants	Analyses for integration	Objective	Reference
Cabernet Sauvignon	*A. tumefaciens*[1]	Kanamycin (10 in liquid culture) Kanamycin (up to 50 in plates)	No plants obtained	-	Protocol development	BARIBAULT *et al.* 1989
Cabernet Sauvignon, Chardonnay, Grenache, Riesling	*A. tumefaciens*	Kanamycin (100)	Data not shown	7 (not indicated)	Protocol development	HUANG and MULLINS 1989
Sultana, Cabernet Sauvignon	*A. tumefaciens*	Kanamycin (10 to 25)	No plants obtained	-	Protocol development	BARIBAULT *et al.* 1990
Grenache	*A. rhizogenes*	Kanamycin (25)	No plants obtained	-	Protocol development	GUELLEC *et al.* 1990
Cabernet Sauvignon, Chardonnay	*A. tumefaciens*	Kanamycin (0 to 25)	No plants obtained	-	Protocol development	MULLINS *et al.* 1990
Thompson Seedless, French Colombard	*A. tumefaciens*	Kanamycin (3 to 7)	No plants obtained	-	Protocol development	COLBY *et al.* 1991
Koshusanjaku	*A. rhizogenes*	Kanamycin (50)	12	12 (Southern Blot+)	Protocol development	NAKANO *et al.* 1994
3 seedless grape selections	Biolistic+ *A. tumefaciens*	Kanamycin (First 6 w 20 and next 6 w 40)	14 (results of the three cultivars shown together)	14 (PCR+)	Protocol development	SCORZA *et al.* 1995
Chardonnay	*A. tumefaciens*	Paromomycin (5 to 20)	50	46 analyzed/30 (GUS+)	Protocol development	MAURO *et al.* 1995
Superior Seedless	*A. tumefaciens*	Kanamycin (50 to 500) Hygromycin (15 to 25) Basta (1 to 10)	Hygromycin (25) 86 Basta (10) 67	60 (PCR+ and Southern Blot+) 42 (PCR+)	Protocol development	PERL *et al.* 1996
Thompson Seedless	Biolistic+ *A. tumefaciens*	Kanamycin (40, 6 w after co-cultivation)	13			SCORZA *et al.* 1996

				13 (PCR+ and Southern Blot+)	Protocol development	
Russalka	*A. tumefaciens*	Kanamycin (100 directly after 2 months of co-culture or a monthly stepwise of 12.2, 25, 50)	Data not shown	Data not shown	Protocol development	GÖLLES *et al.* 1997
Sultana	Biolistic+ *A. tumefaciens*	Kanamycin 1 (30, following co-cultivation and 8-10 w later, 100 or stayed at 50). Kanamycin 2 (50, 3 w after co-cultivation and, 2.5-3 w later, 100) Higromycin (25)	63 12 12	4 (GUS+ and Southern Blot+) 11 (“) 11 (“)	Protocol development	FRANKS *et al.* 1998
Russalka	*A. tumefaciens*	Kanamycin (75 or 100)	Data not shown	140 (PCR+)	Protocol development	GÖLLES *et al.* 1998
Dornfelder, Müller-Thurgau, Riesling	*A. tumefaciens*	Kanamycin (100 and, 4w later, 50)	Data not shown	Data not shown	Protocol development	HARST *et al.* 2000a
Dornfelder, Riesling, Müller-Thurgau	*A. tumefaciens*	Kanamycin (100 solid medium, 100 and, after 4 w, 50 in liquid medium)	Data not shown	Data not shown	Protocol development	HARST *et al.* 2000b
Neo Muscat	*A. tumefaciens*	Kanamycin (50)	More than 20	9 analyzed/7 (PCR+) 5 (Southern Blot+)	Disease resistance	YAMAMOTO *et al.* 2000
Cabernet Sauvignon, Shiraz, Chardonnay, Riesling, Sauvignon Blanc, Chenin Blanc, Muscat Gordo Blanco	*A. tumefaciens*	Kanamycin (100)	Chardonnay 52 Cabernet Sauvignon 136 Sauvignon Blanc 23 Chenin Blanc 57 Muscat Gordo Blanco 9 Shiraz 161 Riesling 19 Semillon 0 Pinot noir 0	7 (Southern Blot+) 31 (“) 5 (“) 19 (“) 1 (“) 45 (“) 2 (“) - -	Protocol development and comparison of transformation efficiency of different cultivarscultivars	IOCCO *et al.* 2001
Russalka	*A. tumefaciens*	Kanamycin (100, 2 months after co-cultivation, or 25 after co-cultivation with a monthly	15			GUTORANOV *et al.* 2001

		stepwise)		2 (PCR+)	Protocol development	
Chardonnay, Shiraz, Danuta, Portan	*A. tumefaciens*	Kanamycin (100 to 150 in 3-4 w)	Portan 20 Shiraz 8 Danuta 0 Chardonnay 0	16 (PCR+ and Southern Blot+) 4 (“) - -	Protocol development	TORREGROSA *et al.* 2002
Silcora, Thompson Seedless	*A. tumefaciens*	Kanamycin (25 the first 30 d, then 50 the next 30 d, and finally 75 the last 30 d)	50	Silcora 10 tested/8 (Southern Blot+). 2 tested/ 2 (RT-PCR+) Thomson Seedless 5 tested/3 (Southern Blot+). 1 tested/ 1 (RT-PCR+)	Protocol development	MEZZETTI *et al.* 2002
Pusa Seedless, Beauty Seedless, Perlett, Nashik	*A. tumefaciens*	Kanamycin (20)	Data not shown	Data not shown	Protocol development	DAS *et al.* 2002
Chardonnay	Biolistic	Kanamycin (10 and, 4 w later, 15)	260	148 analyzed/71 (PCR+) 83 (DBH+)	Protocol development	VIDAL *et al.* 2003
Thompson Seedless, Chardonnay	*A. tumefaciens*	Kanamycin (100)	Thomson Seedless 36 Chardonnay 15	32 (PCR+) 11 (“)	Disease resistance	AGÜERO *et al.* 2005
Seyval Blanc	*A. tumefaciens*	Kanamycin (100)	Data not shown	Data not shown	Disease resistance	BORNHOFF *et al.* 2005
Nebbiolo, Blaufränkisch, Lumassina	*A. tumefaciens*	Kanamycin (50, one month after co-cultivation, and increasing to 100 since the second month of culture)	Nebbiolo 40 Lumassina 1 Blaufrankisch 2	33 (PCR+ and RT-PCR) 40 (Southern Blot+) (22 ELISA+) 1 (“) 1 (“) (ELISA not tested) 2 (“) 2 (“) (ELISA not tested)	Protocol development with GFLV resistance genes	GAMBINO *et al.* 2005
Red Globe	*A. tumefaciens*	Paramomycin (Stepwise 5 to 20) Kanamycin (Between 40 and 100)	Data not shown	Data not shown	Protocol development	WANG *et al.* 2005
Thompson Seedless	*A. tumefaciens*	Kanamycin (25, 50, 75, 100, or 150)	795	Data not shown	Protocol development	LI *et al.* 2006

Chardonnay, Thompson Seedless	*A. tumefaciens*	Kanamycin (100)	Data not shown	Thomson Seedless 191 GUS+ (Fluorescence) 180 PGIP or GFP+ (Radial diffusion assay and fluorescence) 173 GUS and PGIP or GFP+ Chardonnay 41 GUS+ (Fluorescence) 39 PGIP or GFP+ (Radial diffusion assay and fluorescence) 37 GUS and PGIP or GFP+	Protocol development	AGÜERO *et al.* 2006
Chardonnay	Biolistic	Kanamycin (10 and, 4 w later, 15)	19	19 (PCR+) 17 (Southern Blot+) 8 (RT-PCR+) for mag2 9 (“) for MSI99	Disease resistance	VIDAL *et al.* 2006
Thompson Seedless	*A. tumefaciens*	Kanamycin (16)	Data not shown	18 (PCR+) 4 randomly selected (Southern Blot+)	Protocol development	DUTT *et al.* 2007
Thompson Seedless	*A. tumefaciens*	Kanamycin (100 and, 30 d later, 70)	25	18 (PCR+)	To produce transgenic plants without marker genes	DUTT *et al.* 2008
Thompson Seedless	*A. tumefaciens*	Hygromycin (3,6,9,12 or a stepwise selection with the same concentration)	27	19 (PCR+) 9 analyzed/5 (Southern Blot+)	Protocol development and to find the optimal concentration of hygromycin	FAN *et al.* 2008
Sugraone, Crimson Seedless	*A. tumefaciens*	Kanamycin (5, 10, 20, 30, 40, 50)	Crimson Seedless 28 (kan 20) Sugraone 22 (kan 50)	26 (PCR+) 21 (“)	Protocol development and to find the optimal concentration of Kan	LÓPEZ-PÉREZ *et al.* 2008
Thompson Seedless, Merlot, Shiraz	*A. tumefaciens*	Kanamycin (20) Kanamycin (50)	Data not shown	Thomson Seedless 17 analyzed/17 (Southern Blot+)	Protocol deveolpment	LI *et al.* 2008

Centennial Seedless	*A. tumefaciens*	Kanamycin (10)	45	39 (PCR+) 4 analyzed/4 (Southern Blot+)	Cold resistance	JIN *et al.* 2009
Pusa Seedless	*A. tumefaciens*	Hygromycin (10 the first 20 d, then 15 or 20) Hygromicin (25)	Data not shown	7 analyzed/7 (PCR+) for hpt 4(PCR+) for pGL2 4 analyzed/4 (Southern Blot+) 5 analyzed/4 (RT-PCR+) 4 analyzed/4 (Western Blot+)	Disease resistance	NIRALA *et al.* 2010
Chardonnay	Biolistic	Data not shown	Data not shown	28 (PCR+)	Disease resistance	ROSENFIELD *et al.* 2010
Thompson Seedless	*A. tumefaciens*	Kanamycin (100)	71	2 (ELISA+)	Disease resistance	DHEKNEY *et al.* 2011
Albariño	*A. tumefaciens*	Kanamycin (50)	52	7 (PCR+) 5 analyzed/4 (Southern Blot+)	Disease resistance	GAGO *et al.* 2011
Crimson Seedless	*A. tumefaciens*	Hygromycin (5, 2 w after co-culture and, after 2 w more, 10)	Data not shown	Data not shown	Disease resistance	NOOKARAJU and AGRAWAL 2012
Thompson Seddless	*A. tumefaciens*	Kanamycin (25) Kanamycin (50) Kanamycin (75) Kanamycin (100) Kanamycin (150)	351	16 (PCR+) 17 (“) 32 (“) 28 (“) 18 (“)	Protocol development and disease resistance	ZHOU *et al.* 2014
Sugraone	*A. tumefaciens*	Kanamycin (50 after 10 days of the co-culture)	26	2 analyzed/2 (Southern Blot+) 2 analyzed/2 (RT-PCR+)	Disease resistance	DABAUZA *et al.* 2015
Chardonnay	*A. tumefaciens*	Hygromycin (10)	69	32 (Southern Blot+)	Protocol development and disease resistance	DAI *et al.* 2015
Thompson Seedless	*A. tumefaciens*	Kanamycin (not shown)	870	Data not shown. Analyses of random plants	Disease resistance	LI *et al.* 2015
Chardonnay, Shiraz, Cabernet Sauvignon, Malian, Shalistin,	*A. tumefaciens*	Kanamycin (100)	Data not shown			RINALDO *et al.* 2015

Pinot Noir				Data not shown	Study of anthocyanins in transgenic grapevines	
Brachetto	*A. tumefaciens*	Kanamycin (150 after 4 w)	12	6 analyzed/6 (PCR+)	Cisgenesis protocol	DALLA-COSTA *et al.* 2016

[1] *A. tumefaciens* classified now as *Rhizobium radiobacter*

Table 2. Transgenic reports focused on the incorporation of genes related with fungus and bacterial resistance in grapevine.

Cultivar	Gene(s)/Protein(s)	Goal	References
Thompson Seedless	Shiva-1 (lytic peptide gene)	Bacterial resistance	SCORZA *et al.* 1996
Chardonnay, Chancelor and Merlot	n.d. Chitinase (Trichoderma endochitinase)	Powdery mildew resistance	KIKKERT *et al*. 1997
Riesling and Dornfelder	n.d. Chitinase and n.d. glucanase n.d. Chitinase and n.d. Ribosome inactivation protein (RIP)	Disease resistance	HARST *et al*. 2000
Merlot and Chardonnay	*ThEn-42* (endochitinase gene from *Trichoderma harziaru*)	Powdery mildew and Botrytis buch rot resistance	KIKKERT *et al.* 2000 & 2009
Neo Muscat	*RCC2* (Rice Chitinase)	Fungal resistance to powdery mildew	YAMAMOTO *et al.* 2000
Chardonnay	*mag2* (Lytic peptide) *MSI99 (*Sintetic lytic peptide) PGL (Peptidyl-glycine-leucine)	Fungal resistance	VIDAL *et al.* 2003 & 2006
Thompson Seedless and Chardonnay	*pPgip* (Pear Polygalacturonase-inhibiting protein gene)	Enhance resistance to Botrytis	AGÜERO *et al*. 2005
Seyval blanc	n.d. Chitinase and ribosome inactivation protein (RIP from *Hordeum vulgare*)	Antifungal proteins for resistance for *Uncinuka necator* and *Plasmopara viticola*	BORNHOFF *et al.* 2005
Thompson Seedless	*STS* (Stilbene syntase from *Vitis reticulata*)	Phytoalexin for fungal resistance	FAN *et al*. 2008
Pusa Seedless	*Chil1* (Rice Chitinase)	Fungal resistance to powdery mildew	NIRALA *et al.* 2010
Chardonnay	*mag2* and *PGL* (Lytic peptide and peptidil-glycine- leucine respesctively)	Fungal resistance to powdery mildew and crown gall.	ROSENFIELD *et al.* 2010

Thompson Seedless	*Vvtl-1 (Vitis vinifera thaumatin-like protein)*	Fungal resistance for powdery mildew and black rot. Also resistance to sour-bunch rot (bacteria)	DHEKNEY *et al.* 2011
Thompson Seedless	PGIP signal peptide with a cecropin derived lytic domain	Pierce's disease resistance	DANDEKAR et al. 2012
Crimson Seedless	*Chi1* (Chitinase from scab-infected Sumai-3 wheat) *β-1,3-glucanase* (from same wheat)	Anti-fungal genes for increased tolerance to downy mildew fungus	NOOKARAJU and AGRAWAL 2012
Sugraone	*VstI* (Grapevine stilbene syntase)	Enhanced fungal resistance to grey mould (*B. cinerea*)	DABAUZA *et al.* 2015
Chardonnay	*VpSTS* (*Vitis pseudoreticulata* stylbene sintase)	Powdery mildew resistance	DAI *et al.* 2015
Thompson Seedless	LIMA-A (Sintetic gene encoding lythic peptide)	Improve Pierce's disease resistance	LI *et al.* 2015
Thompson Seedless	ech42 (Endochitinase) ech33 (Endochitinase) nag70 (N-acetyl-b-Dhexosaminidase gene)	Increase resistance to Botrytis cinerea and Erysiphe necátor	RUBIO *et al.* 2015
Red Glove	*VpPR4-1* (Pathogenesis-related protein from *Vitis pseudoreticulata*)	Improve powdery mildew resistance	DAI *et al.* 2016

Table 3. Transgenic reports focused on the incorporation into grapevine of genes related with resistance to viruses and other pathogens.

Cultivar	Gene(s)/Protein(s)	Goal	References
Chardonnay	GFLV CP (Grapevine FanLeaf Virus Coat Protein)	Grapevine FanLeaf Virus resistance.	MAURO *et al.* 1995
Thompson Seedless	TomRSV-CP (Tomato RingSpot Virus Coat Protein) Shiva-1 (lytic peptide gene)	Virus and bacterial resistance	SCORZA *et al.* 1996
Rusalka	GFLV CP (Grapevine FanLeaf Virus Coat Protein) ArMV CP (Arabis Mosaic Virus Coat Protein) GVA CP (Grapevine Virus A Coat Protein) GVB CP (Grapevine Virus B Coat Protein)	Resistance to Grapevine FanLeaf Virus, Arabis Mosaic Virus, Grapevine Virus A and B	GÖLLES *et al.* 1997 & 1998
Superior Seedless	MP (Movement Protein)	Grapevine Virus A and B resistance	MARTINELLI *et al.* 1998
Rusalka	GFLV CP (Grapevine FanLeaf Virus Coat Protein)	Grapevine FanLeaf Virus resistance	TSVETKOV *et al.* 2000
Rusalka	GFLV CP (Grapevine FanLeaf Virus Coat Protein)	Grapevine FanLeaf Virus resistance	GUTORANOV *et al.* 2001
Nebbiolo, Blaufränkisch and Lumassina	GFLV CP (Grapevine FanLeaf Virus Coat Protein)	To obtain resistance to Grapevine FanLeaf virus	GAMBINO *et al.* 2005
Sultana	*CYP79A* and *Cyp71E1* (Encoding cytochrome p450 from *Sorghum*) *sbHMNGT* (UDPG glucosyltransferase-encoding from *Sorghum*)	Phylloxera resistance	FRANKS *et al.* 2006
Arich Dressé	IR MPc GFLV (Inverted Repeat Silencing Movement Protein from GFLV)	Grapevine FanLeaf Virus resistance	JARDAK-JAMOUSSI *et al.* 2009
Chardonnay	*pART27-42* (RNA interference silencing a conserved root-knot nematode effector gene *16D10*) *pART27-271*	Root-knot nematodes resistance	YANG *et al.* 2013

Table 4. **Transgenic reports focused on different breeding objectives in grapevine, with the aim of increasing yield by increasing the number of berries or the tolerance of abiotic stresses, particularly low temperatures.**

Cultivar	Gene(s)/Protein(s)	Goal	References
Cabernet Franc	Fe-superoxide dismutase	Freezing tolerance	ROJAS *et al*. 1997
Rusalka	Arf 11 (Antifreeze protein) Arf 62 (Antifreeze protein) Arf 75 (Antifreeze protein) B5 (Antifreeze protein)	Cold resistance	GUTORANOV *et al*. 2001
Vitis vinifera (seedless cultivar)	PPOa (Polyphenol oxidase antisense) UFGT (UDP:flavonoid 3-*O*-glucosyltransferase)	Reduction of PPO levels (browning of damaged plant tissues), berry color	THOMAS and SCOTT 2001
Thompson Seedless and Silcora	*DefH9-iaaM* (Protein that increases IAA formation)	Increased number of flowers and berries	MEZZETTI *et al*. 2002 and CONSTANTINI *et al*. 2007
Portan	*VvAdh2* (*Vitis vinifera* alcohol dehydrogenase)	Abiotic stress resistance	TESNIERE *et al.* 2006
Centennial Seedless	*AtDREB1b* (Dehydration response element binding is a cold-inducible transcription factor in *Arabidopsis thaliana*)	Cold resistance	JIN *et al*. 2009
Brachetto	*VvPIP2;4N* gene (PIP-type aquaporin gene)	Water stress resistance	PERRONE *et al*. 2012

4. Resultados y Discusión

La vid es un cultivo muy importante a nivel mundial, con una producción de 76,5 millones de toneladas y un valor de 44 billones de dólares en 2013 (FAOSTAT 2013). Esta producción se ve mermada a consecuencia de distintos estreses de tipo biótico y abiótico, entre los primeros, son numerosos los virus que la infectan (Martelli 2014). Es por ello importante la utilización de plantas sanas para la propagación y conservación de este cultivo de propagación vegetativa. Según la Comisión directiva 2005/43/EC por la que se modifican los anexos del Consejo Directivo 68/193/EEC el material de vid debe estar libre de los siguientes virus: virus del entrenudo corto (GFLV), virus del enrollado 1 (GLRaV-1), virus del enrollado 3 (GLRaV-3), virus del jaspeado (GFkV) y virus del mosaico del arabis (ArMV).

Existen distintas metodologías para el saneamiento de plantas infectadas con virus, siendo la más utilizada, el cultivo de meristemos combinado o no con termoterapia (Bota et al. 2014, Hancevic et al. 2015). Otras metodologías de interés son la embriogénesis somática (Gambino et al. 2006, Peiró et al. 2015) o la quimioterapia (Guta et al. 2010, Skiada et al 2013). Sin embargo, esta última suele afectar negativamente el desarrollo de las plantas. En un trabajo previo realizado en el grupo de investigación donde se ha llevado a cabo esta tesis, se evaluó la utilización de la embriogénesis somática para el saneamiento de una variedad de vid infectada con virus, utilizando semillas cortadas como material de partida (Peiró et al. 2015). Con este tipo de explante se obtuvo una alta tasa de conversión de embriones en plantas en un corto periodo de tiempo. Este resultado nos condujo a evaluar la respuesta embriogénica a partir de este tipo de explante en otras variedades de vid entre las que se incluyen las siguientes: 'Airén', 'Cabernet Franc', 'Cabernet Sauvignon', 'Godello', 'Mencía', 'Merlot', 'Monastrell', 'Petit Verdot', 'Pinot Blanc', 'Pinot Gris', 'Pinot Meunier', 'Pinot Noir', 'Syrah', 'Tempranillo', 'Valencí Blanc' y 'Verdil', seis de ellas infectadas o multiinfectadas por los virus GFLV, GLRaV-3 y GFkV. Para la inducción de la embriogénesis se compararon dos medios de cultivo que diferían en la concentración del regulador del crecimiento tidiazurón (TDZ) (0.9 µM y

0.45 µM) siendo, en la mayoría de los casos, la concentración más elevada la que una mayor tasa de embriogénesis somática indujo. El hecho de que a los dos meses de cultivo ya se obtuviera una respuesta embriogénica en todas la variedades empleadas, indica la rapidez de este protocolo respecto a otras metodologías previamente utilizadas donde se necesitaban al menos cinco meses para obtener embriones (Gambino et al. 2006 & 2009, Borroto-Fernández et al. 2009). Es la primera vez que se publica la inducción de la embriogénesis en las variedades 'Airén', 'Monastrell', 'Petit Verdot', 'Pinot Blanc', 'Verdil', 'Godello' y 'Valencí Blanc'. En este trabajo también se ha observado germinación a partir de algunas semillas cultivadas y aunque este no era el objetivo del ensayo, puede resultar de interés su uso en programas de mejora convencional. En cuanto a las variedades infectadas, los porcentajes de saneamiento variaron entre el 69% que se obtuvo en 'Tempranillo', infectado con el GFLV, al 100% en 'Cabernet Franc' y 'Valencí Blanc' infectados ambos con el GFkV y el GLRaV-3. Se confirma pues la utilidad de esta metodología para el saneamiento de vid. El virus GFkV no se detectó en ninguna de las plantas analizadas, resultado esperable porque no se transmite por semilla (Digiaro et al. 1999), sin embargo, el GFLV y el GLRaV-3 se encontraron en algunas de las plantas regeneradas. La transmisión a través de semilla del GFLV se ha descrito en Lazar et al. (1990), pero hasta el momento no se ha descrito la transmisión por semilla del GLRaV-3 (Digiaro et al. 1999, Gasparro et al. 2016). Teniendo en cuenta que este cultivo presenta una alta heterocigosidad, entre las plantas saneadas se tienen que seleccionar aquellas regeneradas a partir de tejido materno de la semilla, pues sus diferentes capas desarrollan del tejido esporofítico del óvulo, mientras que el embrión y las células del saco embrionario fusionan con las células espermáticas dando lugar al desarrollo del embrión y del endospermo, respectivamente (Royo et al. 2016). De los dos ensayos realizados en este trabajo, en el primero se utilizaron los seis microsatélites recomendados por la OIV para identificación varietal (VVS2, VVMD5, VVMD7, VVMD27, VrZAG62 y VrZAG79; This et al. 2014) obteniéndose el mismo perfil molecular que las plantas de partida en un 10% de las plantas regeneradas ("true-to-type"). En el segundo ensayo se incrementó el número de marcadores SSR a nueve (VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79, VVMD25, VVMD28 y VVMD32) con un resultado similar. Por tanto, aproximadamente el 10% del material obtenido puede ser empleado, bien para su conservación o bien para su multiplicación. Este porcentaje, que puede parecer bajo, es suficiente para lograr el objetivo de saneamiento, ya que a partir de una planta saneada son numerosos los clones que pueden obtenerse en un periodo relativamente corto de tiempo como muestran los resultados del Capítulo 2. La eficiencia de saneamiento de este trabajo no puede ser comparada con otros en los que se emplea la embriogénesis somática utilizando ovarios y/o anteras como material de partida para el saneamiento de vid, porque en ninguno de los publicados (Goussard et al. 1991, Gambino et al. 2006, Borroto-Fernández et al.

2009) se han analizado las plantas obtenidas para comprobar que son genéticamente iguales a la planta madre.

Teniendo en cuenta el alto coste de establecer un viñedo es muy importante seleccionar el mejor material, por lo que la multiplicación de éste a través de la micropropagación de material libre de virus puede ser de interés (Capítulo 2). En el caso de la vid se han publicado algunos trabajos de micropropagación, pero sin mucho éxito (Zatiko & Molnar 1985, Mathre et al. 2000). En la multiplicación de este cultivo suelen presentarse dificultades como la vitrificación de las plantas o problemas en su correcto desarrollo (Mhatre et al. 2000, Alizadeh et al. 2010) y, además, es habitual que haya un efecto de la composición mineral del medio de cultivo, así como de los reguladores de crecimiento empleados (Skiada et al. 2010, Dev et al. 2016). En este trabajo, se han utilizado plantas libres de virus de la variedad 'Monastrell' obtenidas en un proyecto anterior y se han conseguido altas tasas de multiplicación. Se ha estimado que se pueden obtener con este protocolo hasta 10000 plantas tras siete meses desde el inicio del cultivo. En este ensayo se comparó el rendimiento tras utilizar dos composiciones minerales en el medio de cultivo; éste se duplicó al utilizar las sales de 'Lloyd and McCown woody plant salts' (medio W) respecto de las de 'Murashige and Skoog' (medio B) a un medio de concentración de los macronutrientes. Los brotes inducidos elongaron sin dificultad y no se observó vitrificación como se ha descrito en Alizadeh et al. (2010). Esta diferencia de resultados puede deberse a la utilización de otro genotipo hasta ahora no empleado, a la composición del medio de cultivo que no es similar o a la transferencia en nuestro trabajo de los brotes inducidos en medio con Bencilaminopurina (BAP) a medio de cultivo sin reguladores del crecimiento para facilitar el desarrollo. El rendimiento también resultó ser mucho más alto que, por ejemplo, el obtenido en el trabajo de De Carvalho-Silva et al. (2012) que, usando una concentración de BAP menor y con el mismo tiempo de cultivo (90 días), obtuvieron entre 1.9 y 2.8 nudos por explante frente a los 21.75 obtenidos en este trabajo. Una muestra de las plantas obtenidas se aclimató en invernadero y fue transferida a campo satisfactoriamente. El medio MW utilizado para el cultivo de los brotes micropropagados ha sido evaluado y ha resultado útil en otras variedades de vid (Capítulo 3).

La conservación de germoplasma tanto *in situ* como *ex situ* son necesarias y complementarias para la preservación de la diversidad genética que representa una fuente de variabilidad muy importante para poder llevar a cabo programas de mejora (Vasanth & Vivier 2011). La conservación *in vitro* es una metodología que complementa a las colecciones en campo y que permite almacenar un gran número de plantas en un espacio reducido, además de facilitar el intercambio de germoplasma o la transferencia de material para otros propósitos (Santana et al. 2008, Ray & Bhattacharya 2010, Leao

& Motoike 2011), pero no es muy utilizada en vid donde sí existen distintas colecciones que albergan un gran número de accesiones en parcelas de cultivo. Entre las más importantes: 'The Domaine de Vassal' en Montpellier (Francia) con 2300 variedades incluyendo especies silvestres, portainjertos, híbridos y mutantes, el 'Julius Khün Institute' en Siebeldingen (Alemania) con unas 3000 accesiones, y 'La colección de vides de El Encín' en el IMIDRA (Alcalá de Henares, España) con 3532 accesiones entre las que hay 852 portainjertos, 69 híbridos interspecíficos, 111 *Vitis* spp., 1852 variedades de *V. vinifera*, de las cuales 1178 son vinícolas y 674 de mesa, y 648 *V. vinifera sylvestris.*

Para iniciar un banco de germoplasma *in vitro* se necesita disponer del material vegetal que represente la mayor variabilidad posible (reservorio de genes que puedan hacer frente a nuevos patógenos o a las adversidades del clima), comprobar el estado sanitario, obtener planta libre de virus si es necesario, y establecer las condiciones adecuadas que permitan mantener el germoplasma en las mejores condiciones posibles. En esta tesis se han llevado a cabo actuaciones en estos tres ámbitos (Capítulos 1-4). En concreto, en el Capítulo 3, se muestran los ensayos realizados para evaluar el crecimiento y seleccionar los medios de cultivo para la conservación de 16 variedades de vid que incluyen variedades de interés comercial ('Airén', 'Chardonnay', 'Garnacha Blanca', 'Moscatel de Alejandría', 'Moscatel de Grano Menudo', 'Pedro Ximénez', 'Pinot Blanc', 'Pinot Gris', 'Sauvignon Blanc' y 'Tempranillo'), minoritarias ('Chelva', 'Verdil', 'Bobal', 'Valencí Blanc' y 'Valencí Negre) y en peligro de extinción ('Esclafacherre'). Se ha comparado el crecimiento en el medio MW desarrollado anteriormente, y en este medio con modificaciones (reducción de la concentración de sacarosa a la mitad y eliminación de regulador de crecimiento Ácido Indolbutírico (IBA)) para seleccionar el más óptimo pudiendo alargar el tiempo entre los subcultivos y reduciendo los costes de mantenimiento. Se han obtenido diferencias de crecimiento entre variedades y medios de cultivo, lo que es esperable puesto que en cultivo *in vitro* es diferente la respuesta en función del genotipo (García-Águila et al. 2004, Hassanen et al. 2013). Tras la evaluación de distintos parámetros de crecimiento vegetativo (porcentajes de brotación, porcentajes de plantas con hojas, formación de callo, longitud de las plantas a diferentes tiempos de cultivo y número de hojas por planta) y desarrollo radicular (índice radicular), se ha considerado adecuado el medio MW para el crecimiento y mantenimiento *in vitro* de las variedades 'Tempranillo', 'Bobal', 'Valencí Negre', 'Valencí Blanc', 'Esclafacherre', 'Garnacha Blanca', 'Moscatel de Alejandría', 'Moscatel de Grano Menudo', 'Pinot Blanc' y 'Pinot Gris', ya que en este medio se desarrollan correctamente y enraizan sin formar callo a una velocidad que permite mantenerlas en cultivo *in vitro* activo durante aproximadamente 2-3 meses sin necesidad de cambiarlas de medio. Por otra parte, reducir la concentración de sacarosa a la mitad en el medio de cultivo ha sido eficaz para alargar el tiempo entre subcultivos al reducir el crecimiento en los

cultivares ‘Airén’, ‘Chardonnay’, ‘Chelva’, ‘Pedro Ximénez’ y ‘Verdil’ y no ha afectado ni a la brotación ni a la formación de raíces (salvo en la variedad ‘Pedro Ximenez’). Por otra parte, la supresión del IBA en el medio de cultivo se ha traducido en la ausencia de callosidad que se producía en la base de las plantas en las variedades ‘Chardonnay’, ‘Chelva’, ‘Moscatel de Alejandría’, ‘Pinot Gris’ y ‘Sauvignon Blanc’ en el medio MW. También, la variedad ‘Chelva’ ha reducido su crecimiento al eliminar la auxina.

Otra metodología relacionada con la conservación que se está evaluando actualmente es la crioconservación de meristemos para, además de conservar los genotipos activamente, tener duplicados crioconservados (Capítulo 4). Se ha validado la utilidad del protocolo descrito en Gisbert et al. (2015) para *Bituminaria bituminosa* en la variedad ‘Esclafacherre’. Este protocolo utiliza la gotita congelada y la solución de crioconservación PVS2 (Plant Vitrification Solution 2). La incubación durante 50 minutos en la solución PVS2 previamente a la introducción de los meristemos (precultivados en medios ricos en azúcar) en nitrógeno líquido permite la recuperación de plantas. Como la solución de crioconservación puede resultar muy tóxica, se está evaluando el tiempo de incubación en distintos cultivares para optimizar el rendimiento (número de plantas recuperadas tras la crioconservación).

En el Capítulo 4 también se hace referencia a otros trabajos necesarios para establecer bancos *in vitro*, aquellos encaminados a la identificación del material a conservar y que permiten detectar variantes, homonimias y sinonimias que son muy frecuentes en el cultivo de la vid (Stajner et al. 2008). Estos análisis, que se realizan principalmente utilizando marcadores de tipo microsatélite, nos permiten descartar materiales que se consideran distintos pero no lo son y no perder materiales que se consideran equivalentes, pero que tampoco lo son. En concreto en este capítulo se publica por primera vez el perfil de marcadores microsatélites de la variedad ‘Esclafacherre’, una variedad que se encuentra actualmente en peligro de extinción pero que era muy común en la Comunidad Valenciana en la época pre-filoxera. También se ha comprobado la identidad de distintas accesiones de la variedad histórica ‘Valencí Blanc’ (Fernández-González et al. 2007, García-Muñoz et al. 2010) observando diferencias en el marcador VVMD32 para las accesiones de la provincia de Alicante y las de otros orígenes lo que puede indicar la existencia de dos variantes que pueden ser estudiadas con mayor profundidad.

Por último, en el Capítulo 5 de esta tesis figura una revisión bibliográfica (Saporta et al. 2016) que se ha realizado con el fin de analizar el estado actual de la aplicación de la transformación genética en vid y sus factores limitantes, haciendo hincapié en los distintos factores relacionados con la

regeneración de plantas a partir de las células transformadas. En este trabajo se ha podido concluir que la mejora de variedades de vid mediante transformación se ha centrado en obtener resistencia a determinados patógenos con algunos resultados prometedores, como una mayor resistencia a nematodos (menos huevos por raíz) utilizando secuencias del gen *16D10* que juega un papel importante estableciendo sitios de alimentación (Yang et al. 2013) o el publicado por Rubio et al. (2015), donde se obtuvo mayor resistencia a *Botrytis cinerea* y *Erysiphe necator* en las plantas transgénicas que expresaban las endochitinasas *ech42*, *ech33* y el gen de resistencia *nag70*, repectivamente. Sin embargo, la mayoría de los trabajos publicados no han conseguido resultados prácticos. Por otra parte, se puede extraer de esta revisión que aunque han utilizado como metodologías de transformación la infección con *Agrobacterium tumefaciens* (nombre actual *Rhizobium radiobacter*) y la biolística, la primera es la más adecuada para obtener plantas que expresen el transgén o transgenes a introducir, mientras que los ensayos con biolística en vid son más exitosos para estudios de expresión transitoria. Dentro de los factores limitantes para la transformación estable, figura el genotipo, que va a determinar en gran medida el éxito en la regeneración a partir de las células transformadas. En diferentes especies se han encontrado varios QTLs (Quantitative Traits Loci) que contienen genes relacionados con la regeneración, lo que indica que son varios genes los que influyen en este proceso y explica las diferencias encontradas en cuanto a la capacidad regenerativa en distintos cultivares de la misma especie (Priyono et al. 2010, Trujillo-Moya et al. 2011, Zhenzhen et al 2015). En el caso de la vid, las variedades 'Chardonnay' y 'Thompson Seedless' han sido las más utilizadas como ocurre en otros cultivos donde se utilizan comúnmente aquellas con mejor aptitud para la regeneración. Además de la capacidad regenerativa intrínseca, cada genotipo muestra una sensibilidad diferente a la cepa de *Agrobacterium*, al agente selectivo que se utiliza en el medio de regeneración y a la toxicidad de las partículas si el ensayo es con biolística. En las variedades de vid es frecuente obtener embriones somáticos aberrantes que no consiguen desarrollarse en plantas normales, incluso en trabajos de regeneración a partir de células no transformadas (López-Pérez et al. 2006, Bharathy & Agrawal 2008) y se han estudiado distintas estrategias de selección con el fin de favorecer la regeneración de plantas transformadas (Zhou et al. 2014, Li et al. 2015). A pesar de que los resultados hasta la fecha no son muy abundantes por las limitaciones anteriormente comentadas, es esperable un incremento de intentos de transformación en vid a causa del mayor conocimiento de la función génica y su regulación, la optimización de protocolos de regeneración y la aparición de nuevas tecnologías para la edición y transferencia de genes (Dutt et al. 2014, Bortesi & Fischer 2015).

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5. Conclusiones

1. La utilización de la embriogénesis somática ha resultado útil para la regeneración de plantas libres de virus a partir de semillas de plantas infectadas con el GFkV, GFLV y GLRaV-3 en las variedades 'Cabernet Franc', 'Godello', 'Merlot', 'Pinot Blanc', 'Tempranillo' y 'Valencí Blanc'. Este tipo de explantes cultivados en el medio EIM2 que contiene el regulador TDZ a una concentración de 0.90 µM ha proporcionado altas tasas de regeneración y conversión de los embriones somáticos en plantas en las 16 variedades utilizadas y en un menor tiempo en comparación con otros protocolos, siendo la primera vez que se publica la inducción de la embriogénesis en las variedades 'Airén', 'Monastrell', 'Petit Verdot', 'Pinot Blanc', 'Verdil', 'Godello', y 'Valencí Blanc'. Alrededor de un 10% de las plantas obtenidas ha mostrado el mismo perfil genotípico que las plantas de partida, es decir han regenerado a partir de los tejidos de la cubierta que son de origen materno. En este trabajo también se ha observado germinación (zigótica) a partir de algunas semillas cultivadas, y aunque este no era el objetivo del ensayo, puede resultar de interés su uso en programas de mejora convencional. Por otra parte, los datos de saneamiento obtenidos confirman que el GFkV no se transmite por semilla (100% de eficiencia de saneamiento) y que los virus GFLV y GLRaV-3 sí se encontraban en las semillas de partida (porcentajes variables de saneamiento).

2. A partir de planta sana de la variedad 'Monastrell' cultivada *in vitro* se ha desarrollado un protocolo de micropropagación con el que se podrían obtener alrededor de 10.000 plantas en siete meses a partir de una planta madre. En el desarrollo del protocolo se ha determinado la influencia de las sales del medio de cultivo en el rendimiento, que se duplica al utilizar las sales de 'Lloyd and McCown woody plant salts' (medio W) respecto de las de 'Murashige and Skoog' (medio B) a un medio de concentración de los macronutrientes con o sin la adición del regulador del crecimiento BAP.

3. El medio MW utilizado para el desarrollo de las plantas obtenidas mediante micropropagación, es adecuado para la brotación, crecimiento y enraizamiento *in vitro* de las variedades evaluadas entre las que se incluyen variedades comerciales ('Airén',

‘Chardonnay’, ‘Garnacha Blanca’, ‘Moscatel de Alejandría’, ‘Moscatel de Grano Menudo’, ‘Pedro Ximénez’, ‘Pinot Blanc’, ‘Pinot Gris’, ‘Sauvignon Blanc’ y ‘Tempranillo’), variedades minoritarias (‘Chelva’, ‘Verdil’, ‘Bobal’, ‘Valencí Blanc’ y ‘Valencí Negre’) y, la variedad en peligro de extinción ‘Esclafacherre’. Modificaciones en el medio de cultivo MW, como la reducción a la mitad de la concentración de sacarosa o la eliminación del IBA disminuyen el crecimiento en algunas variedades a la vez que se reduce la formación de callo en la base al eliminar la auxina en aquellas variedades en las que se presentaba formación de callo. Por tanto, se puede seleccionar para cada variedad aquel medio que permita alargar el tiempo entre subcultivos, minimizando costes.

4. El protocolo descrito en Gisbert et al. (2015) para *Bituminaria bituminosa* que utiliza la gotita congelada y la solución de crioconservación PVS2 ha sido exitoso para la crioconservación de meristemos de la variedad ‘Esclafacherre’, si bien se están realizando otros ensayos para optimizar los rendimientos.

5. El perfil genético obtenido con 15 marcadores de tipo microsatélite en dos accesiones de la variedad ‘Esclafacherre’ (‘Esclafagerres’) y en 14 accesiones de la variedad ‘Valencí Blanc’ de distinto origen, nos ha permitido comprobar que ambas accesiones de ‘Esclafacherre’ son iguales y que su perfil no coincide con ninguna de las variedades disponibles en las bases de datos (>3000), por lo que se correspondería con el de esta variedad en peligro de desaparición; en cuanto a las accesiones de la variedad histórica ‘Valencí Blanc’ este análisis ha permitido comprobar su identidad y separar dos grupos (las accesiones de la zona de Alicante y el resto) que difieren en uno de los marcadores utilizados.

6. La revisión bibliográfica realizada en relación con el estado actual de la transformación genética en vid nos ha llevado a las siguientes conclusiones: 1) la mejora de vid se ha centrado principalmente en la resistencia a estreses bióticos; 2) la capacidad de regeneración es un paso limitante; 3) la mayoría de trabajos con éxito en la regeneración de plantas transgénicas han utilizado las variedades ‘Chardonnay’ y ‘Thompson Seedless’ lo que muestra la influencia del genotipo; 4) en la dificultad para regenerar las plantas transgénicas también contribuyen los antibióticos u otros agentes selectivos utilizados (concentración y tiempo de aplicación); 5) la biolística ha resultado útil en estudios de expresión transitoria pero no parece adecuada para obtener plantas con integración estable

en vid y 6) la transformación mediada por *Agrobacterium* utilizando el gen *nptII* y el uso de kanamicina como agente selectivo ha sido la metodología más empleada para la regeneración de plantas transgénicas de vid.