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Recovering Ancient Grapevine 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 "Valenci Blanc" (or "Beba") and the historic and endangered variety "Esclafagerres" ("Esclafacherres" or "Esclafacherris").

Keywords: Valenci 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 2910 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 *Vitis* International Variety Catalog (VIVC; <http://www.vivc.de/>), supported by Biodiversity and the International Organization of Vine and Wine (OIV), 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% (7714) correspond to *Vitis* interspecific crossing. Around 25% of the cultivars were registered in France (5602), followed by the United States (2401) and Italy (2348) 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 1902 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 is 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”) dominates vineyards worldwide [5]. Considering that most major wine-producing regions could become by 2050 unsuitable for currently grown cultivars [6, 7], the preservation of genetic variability and the selection and/or development of cultivars well adapted to upcoming climate changes are important. The long juvenile period of grapevine makes breeding a slow process; therefore, the knowledge of the raw material and their availability is very important to speed up breeding programs.

1.1. 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 in 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 nineteenth 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 *Vitis vinifera subsp. sylvestris* is a major stake in grapevine preservation.

1.2. Grapevine preservation

The importance of germplasm preservation 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 Kühn 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 7800 accessions *ca* 50 countries, representing 2300 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 3000 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 3532 accessions that are grouped into 852 rootstocks; 69 interspecific hybrids; 111 *Vitis* spp.; 1852 *V. vinifera* varieties, of which 1178 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 is a common strategy, although limited by budget constraints.

Another 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 the 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 ultralow 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].

In grapevine, it is convenient to check by molecular markers that the variety to be stored really corresponds to it because homonymies (similar name for different cultivars) and/or synonymies (different names for a same cultivar) are commonly found. 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. It 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 twentieth 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, the development of protocols adjusted to the variety to be preserved is necessary. For this kind of storage, it is important to choose 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 (reduction 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 solution 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**).

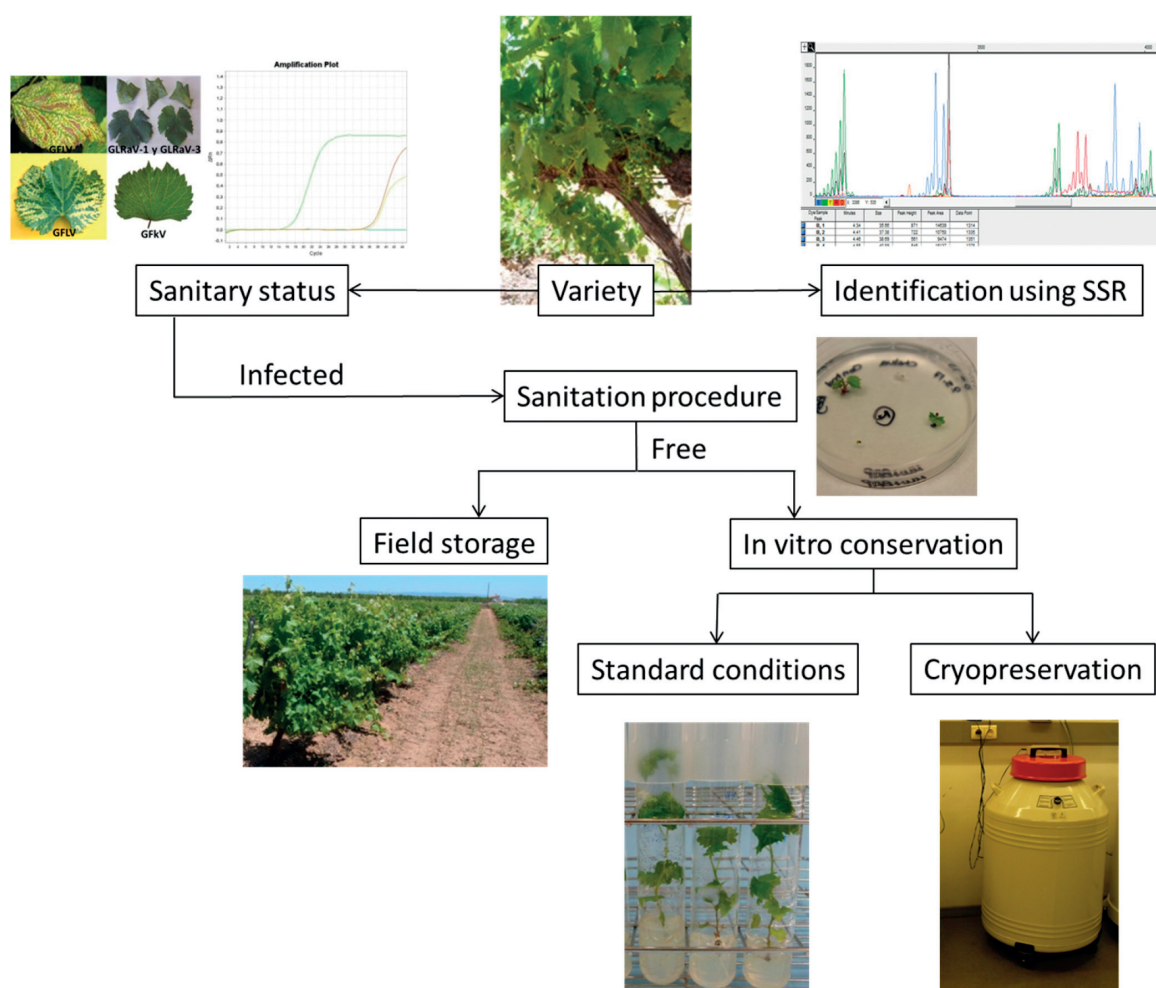


Figure 1. Scheme including the main steps required for germplasm storage.

2. Recovering ancient germplasm at the Comunitat Valenciana: the historic varieties “Valenci Blanc” and “Esclafagerres”

2.1. Richness of grapevine germplasm in the Comunitat Valenciana

The Comunitat Valenciana which includes the provinces of Alicante, Valencia, and Catellón 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 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 grapevine 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 aphids 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 aphids spread to Sagunto, Liria, and Requena [53].

In the context of the research project CGL2015-70843-R, we initiated different approaches in order to contribute in the recovering of ancient varieties in the 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 “Valenci 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.

2.2. The historic varieties “Valenci Blanc” and “Esclafagerres”

The “Valenci 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 it 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 “Valenci” (“Valensi” and “Balansi” 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], the culture of “Grumer” is noted, among other 18 grapevine varieties in the Alicante province and in Albaida and Onteniente (locations nearest to the Alicante province). In the same report, the culture of “Valensi” in Alberique and Enguera (nearest to Valencia City) appeared. Recently, with the name of “Grumer,” we have identified some accessions from the Alicante province that do not correspond to “Valenci” but grouped with “Muscat Istambul” [61]. Lacombe et al. [62] proposed as the origin of “Muscat Istambul” the cross of “Muscat of Alexandria” × “Valenci Blanc” which was confirmed by Mena et al. [63]. Therefore, it could be

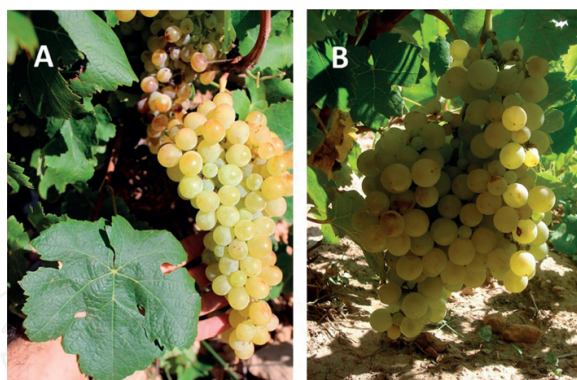


Figure 2. Grapes of “Valenci Blanc” (A) and “Esclafagerres” (B) varieties.

easy to found this homonym. In the VIVC database, 71 synonymies for “Beba” appeared. However, some of these (“Chelva,” “Hebén,” “Mantúo,” “Teta de Vaca,” and “Uva de Planta”) were rejected as they 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 “Valenci” was included in the groups of cultivars with black and white berries. The comparison among the SSR profiles of some accessions of “Valenci” with white grapes (“Valenci Blanc”) and with black grapes (“Valenci Tinto” or “Valenci Negro” in the Comunitat Valenciana) indicates that they are not mutant for berry color but resulting from different crosses. Comparing the SSR profile of the variety “Heben” (or “Gibi”), proposed as parent of “Valenci Blanc,” with the SSR profile of the accession of “Valenci Tinto” hold in the VIVC database, no relationship between them was observed. However, one or two alleles were shared between “Valenci Blanc” and “Valenci Tinto.” Therefore, the unknown parent from “Valenci Blanc” could be the parent of “Valenci 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.

2.3. Determining the SSR profiles

A total of 14 samples of “Valenci 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

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

Allele sizes are expressed as base pairs.

Table 1. SSR profiles for 14 accessions of “Valenci 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.

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 were 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: carboxyfluorescein (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 μ L, using 1.25 μ L of commercial Master Mix PCR Multiplex (Takara Multiplex Hot Short PCR, Takara), 20–40 ng of genomic DNA, 0.1 μ L of Takara Taq Hot Start, and labeled multiplexed SSR primers (from 5.5 to 35.0 μ mol). 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 μ L of an internal size standard (GeneScan™ 500 LIZ, Applied Biosystems) was mixed with 1.00 μ L of PCR product and 10.87 μ L 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 in **Table 1**. Whereas similar SSR profiles were found for both accessions of “Esclafacherre,” some variability is found among “Valenci Blanc” accessions. Among the 15 SSRs 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 homozygosity. We can consider that among the analyzed germplasm, there are two variants of “Valenci 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 “Valenci 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 3265 accessions), those in the International Vitis database (including 3430 accessions), as well as those reported in several publications [62, 63, 69] did not match with any of the included varieties. No matches were 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.

2.4. 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 μ L of crude extract using an Ultraclean Plant RNA isolation kit (Mobio) following the manufacturer's instructions. The real-time multiplex reverse transcription polymerase chain reaction (RT-PCR) was performed for the simultaneous detection of ArMV, GFLV, GFkV, GLRaV-1, and GLRaV-3 using a StepOnePlus thermocycler (Applied Biosystems) and a reaction mixture containing 1 \times AgPath-ID One-step RT-PCR buffer (Ambion) and 1.5 \times AgPath-ID One-step RT-PCR enzyme mix (Ambion); 5 μ L 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°C for 10 min and a denaturation step at 95°C for 10 min, followed by 45 cycles of amplification (95°C, 15 s; 50°C, 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 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, “Valenci Blanc” (sample Vb-Pe0), which resulted in infection with GFkV and GLRaV-3, was sanitized through both meristem culture and somatic embryogenesis. Meristems (n = 35) from plants of “Valenci Blanc” were extracted using a binocular lent and cultured in vitro on plates (90 \times 15 mm) containing the medium MW, selected for “Monastrell” micropropagation [71] supplemented with 6-benzylaminopurine (BAP) at 1.8 μ M. 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 is putatively the cause 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 “Valenci Blanc” were extracted from grapes and disinfected and cut previously to be cultured on the embryogenesis induction medium (EIM2) which contained TDZ (thidiazuron) at 0.9 μ M. Thirteen percent 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**).

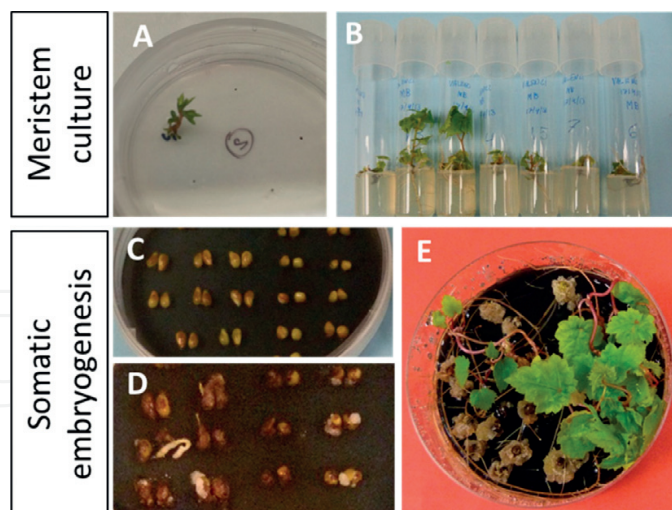


Figure 3. Strategies for virus sanitation of “Valenci Blanc” (accession Vb-Pe0). (A) Meristems cultured on MW supplemented with 6-benzylaminopurine (BAP) at 1.8 μ M and without indole butyric acid (IBA) after 20 days of culture. (B) Plants from meristems cultured in MW medium after 90 days of culture. (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.

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 “Valenci 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.

2.5. In vitro storage

Sanitized plants of “Valenci Blanc” and “Esclafagerres” are maintained in tubes with MW medium in an in vitro culture growth chamber under standard conditions ($25 \pm 2^\circ\text{C}$; 16 h light). The MW medium is adequate to storage a broad spectrum of grapevine cultivars including “Valenci Blanc” and “Esclafagerres” [44]. Both cultivars grew less than 4 cm after 40 days of culture in this medium. 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, the higher the cost and the higher the 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 pretreatment with salicylic acid was performed prior to 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 have been developed and applied to rescue virus-free plants. Among the analyzed materials, the historic varieties “Valenci Blanc” and “Esclafagerres” were sanitized and currently are maintained under in vitro culture conditions. Differences for the microsatellite VVMD32 were found among “Valenci 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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