

Incidence of virus infection in old vineyards of local grapevine varieties from Majorca: implications for clonal selection strategies

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Abstract

Three autochthonous grapevine varieties of Majorca (Spain) were analyzed for the presence of viruses listed by the international certification programs. Enzyme-Linked Immuno-Sorbent Assay (ELISA) screenings were performed in 193 vines from 46 vineyards included in a clonal selection. Virus-free vines were only 6.4%, 9.6% and 11.5%, in Manto Negro, Callet and Moll, respectively. Infections by grapevine leafroll associated viruses (GLRaVs) were ascertained in 71%, 78% and 60% of Manto Negro, Callet and Moll vines, respectively. Each variety was also highly infected by Grapevine fanleaf virus (GFLV) and Grapevine fleck virus (GFkV). The percentage of plants displaying multiple infections was 58.4% in Manto Negro, 63.8% in Callet and 42.6% in Moll. Thus, it was very difficult to identify virus-free clones with suitable agronomic characteristics to be considered as a reference for the grape market. In order to obtain certified propagation material under such conditions of endemic viral infection, sanitation should be the main focus in clonal selection processes. However, the time and financial requirements for proper sanitation process bring to consideration the need to use, at least temporarily, standard multiplication material while certified clones are achieved.

Additional key words: autochthonous varieties, grapevine certification, standard material virus incidence.

Resumen

Incidencia de las infecciones víricas en antiguos viñedos de tres variedades de vid autóctonas de Mallorca: consecuencias sobre las estrategias de la selección clonal

En este estudio se analizó la presencia de las virosis contempladas por las leyes internacionales en tema de certificación en tres variedades de vid autóctonas de Mallorca. Para ello se realizó el test ELISA (enzyme-linked immunosorbent assay) sobre 193 cepas procedentes de 46 viñedos incluidos en un programa de selección clonal. Las cepas que resultaron libres de los virus analizados fueron el 6,4% en Manto Negro, el 9,6% en Callet y el 11,5% en Moll. Los porcentajes de cepas infectadas por los virus asociados al síndrome del enrollado de la vid (GLRaVs) fueron 71% en Manto Negro, 78% en Callet y 60% en Moll. Se detectaron también altas tasas de infecciones para el virus del entrenudo corto infeccioso de la vid (GFLV) y el virus del jaspeado de la vid (GFkV) en las tres variedades. Los porcentajes de cepas sujetas a infecciones múltiples fueron 58,4% en Manto Negro, 63,8% en Callet y 42,6% en Moll. Por tanto fue difícil encontrar cepas que cumpliesen tanto los requisitos sanitarios para la certificación como aquellos agrónomicos necesarios según las exigencias del mercado vitivinícola. En tales condiciones de infecciones víricas endémicas, el saneamiento debería ser el primer objetivo a seguir con el fin de obtener material de propagación certificado. Sin embargo, el tiempo y los recursos económicos necesarios para llevar a cabo el proceso de saneamiento conllevan la necesidad de considerar el uso temporal de material de propagación estándar hasta que se obtengan clones certificados.

Palabras clave adicionales: certificación en *Vitis vinifera*, incidencia de virosis, material estándar, variedades autóctonas.

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Abbreviations used: ArMV (*Arabic mosaic virus*), ELISA (enzyme-linked immunosorbent assay), EU (European Union), GFkV (*Grapevine fleck virus*), GFLV (*Grapevine fanleaf virus*), GLRaV (*Grapevine leafroll associated virus*), RW (*rugose wood*).

Introduction

The main autochthonous grapevine varieties of the Balearic Islands are Manto Negro, Callet and Moll. Manto Negro and Callet have red grapes, while Moll has white grapes. According to the census of 2008, Manto Negro is the most widely cultivated in the archipelago with 20% (309 ha) of the total grape cultivation area; Callet is the third most common variety with 10% of the area while Moll is the sixth one, with 6% (Conselleria d'Agricultura i Pesca, Govern de Les Illes Balears, personal communication). There are two appellations for Majorcan wine: Binissalem-Mallorca and Pla i Llevant. The former is expected to present, at least, 50% Manto Negro grapes for red wines and 50% Moll grapes for white wines. In Pla i Llevant, Callet is the most common and appreciated red variety.

The aromatic profile and production qualities of these varieties have generated interest for future use in other wine growing regions. Each of the three varieties has a distinct aromatic profile, contributing to the wine tipicity (Armero and Alabern, 1990). In addition, Moll is very productive in fertile soils without significant changes in must quality, but occasionally presents low acidity. Manto Negro and Callet are often low in colour intensity and occasionally in sugar content, but show very interesting must quality in poor and shallow soils (Armero and Alabern, 1990; Carambula *et al.*, 2006). The importance of these varieties in the Balearic Islands and the potential for their use in other regions lead to the initiation of a clonal selection process for their characterization, improvement and conservation.

Worldwide, clonal selection is the most common method used to improve grapevine varieties (Walter and Martelli, 1997; Mannini, 2000). In the European Union (EU), genetic and sanitary selections are usually performed simultaneously (Mannini, 2000). Generally, this process consists of several steps: first, a number of old vineyards sited in a given area are selected, and several vines are chosen and evaluated for three to four years. After the initial observation period, the most interesting vines, chosen according to specific requirements for varietal improvement, are grafted and grown in at least two separate fields (homologation fields) where they are further observed for four to five years. Finally, the best clones are homologated. The final aim of a clonal selection process is the achievement of clones free from the most harmful grapevine viruses (certified clones) and possessing varietal identity. At the same time, unique agronomic features of clones of the same

variety are often sought with the purpose of providing market differentiation.

Grapevine fanleaf virus and GLRaVs are worldwide spread in grapevine areas (McKenzie *et al.*, 1996; Andret-Link *et al.*, 2004). Several findings attest the negative influence of these viruses on grape production and must quality, and thus the importance of sanitation by clonal selection process (Goheen, 1989; Walter and Martelli, 1996; Mannini, 2003). According to the Commission Directive 2005/43/EC (OJ, 2005) amending the Annexes to Council Directive 68/193/EEC (OJ, 1968) on the marketing of grapevine propagation material, each member state should ensure the absence of GFLV, GLRaV-1, GLRaV-3, ArMV and GFkV (for rootstocks only) in grapevine nursery plants. The International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICGV) also recommends checking for the presence of GLRaV-2, the viruses linked to Rugose Wood (RW) complex and phytoplasmas (Grapevine Yellows) in grapevine certification programs (Martelli, 2006).

The commerce of standard propagation material is also allowed; at the beginning of this clonal selection, although serological and molecular tests were available, only varietal identity and visual checks for «harmful organisms» were required. However the 2005/43/EC schedules that the member states of UE should provide standard propagation material with major sanitary guarantees. This material does not have any specific identification numbers and propagation material can derive from different plants. Thus, complete traceability is lacking. Despite the ICGV recommendations to use certified plants, the circulation of standard category propagation material is quite common within small growing areas (*i.e.*, provinces and autonomous regions).

The abundance of GFLV, GLRaV-1, GLRaV-2, GLRaV-3 and GFkV within 193 vines belonging to three autochthonous varieties has been studied. The implications of these results for the clonal selection process, the efforts to conserve germoplasm, and vine selection for evaluation in the homologation fields are discussed.

Material and methods

Clonal preselection and criteria for vine choice for homologation field evaluation

Approximately 200 vines per variety were followed from 2001 to 2004 (Cretazzo *et al.*, 2007). Vines were

identified from 39 vineyards of Callet, 33 of Manto Negro and 13 of Moll. The criteria of the choice were a constant production throughout the years, a good equilibrium between vigour and yield, satisfactory sanitary condition and disease resistance, and an adequate grape colour for red varieties. All vineyards were over 20 year old and located in either Binissalem-Mallorca or Pla i Llevant appellation (Fig. 1). According to the objectives of the appellations, minimum requirements were established to choose the candidate clones for including in the homologation step (Table 1). Fourteen clones of Manto Negro, 17 of Callet and 13 of Moll were selected and grafted in 2006 in the homologation fields located in Binissalem-Mallorca and Porreres (Pla i Llevant).

Serological tests

Enzyme-linked immuno-sorbent assays (ELISA) for the detection of GFLV, GLRaV-1, GLRaV-2, GLRaV-3

and GFkV were performed in 2005 and replicated in 2006 to detect viral infections of vines. Antibodies were obtained from commercial sources (below). One hundred and ninety three vines incorporated in the clonal preselection, including all 44 candidate clones, were screened (48 Manto Negro vines collected from 14 vineyards, 83 Callet vines from 21 vineyards and 62 Moll vines from 11 vineyards, see Annex). Furthermore, in 2007, ELISA analysis were performed on 10 replicates of each candidate clone coming from the homologation field of Binissalem-Mallorca, in order to check both virus transmission by graft and sanitary conditions of the experimental field.

For GFLV and GFkV detection, the sampling was performed in May. Each sample consisted of five shoot basal leaves picked around the vine perimeter. For each leaf the terminal part of the petiole and a contiguous portion of limb were excised for the extraction. Plant tissue was homogenized with PBS ($8 \text{ g L}^{-1} \text{ NaCl}$, $0.2 \text{ g L}^{-1} \text{ KCl}$, $0.2 \text{ g L}^{-1} \text{ KH}_2\text{PO}_4$, $1.14 \text{ g L}^{-1} \text{ Na}_2\text{HPO}_4$, $\text{pH} = 7.2\text{-}7.4$), supplemented with 0.2% w/v DIECA

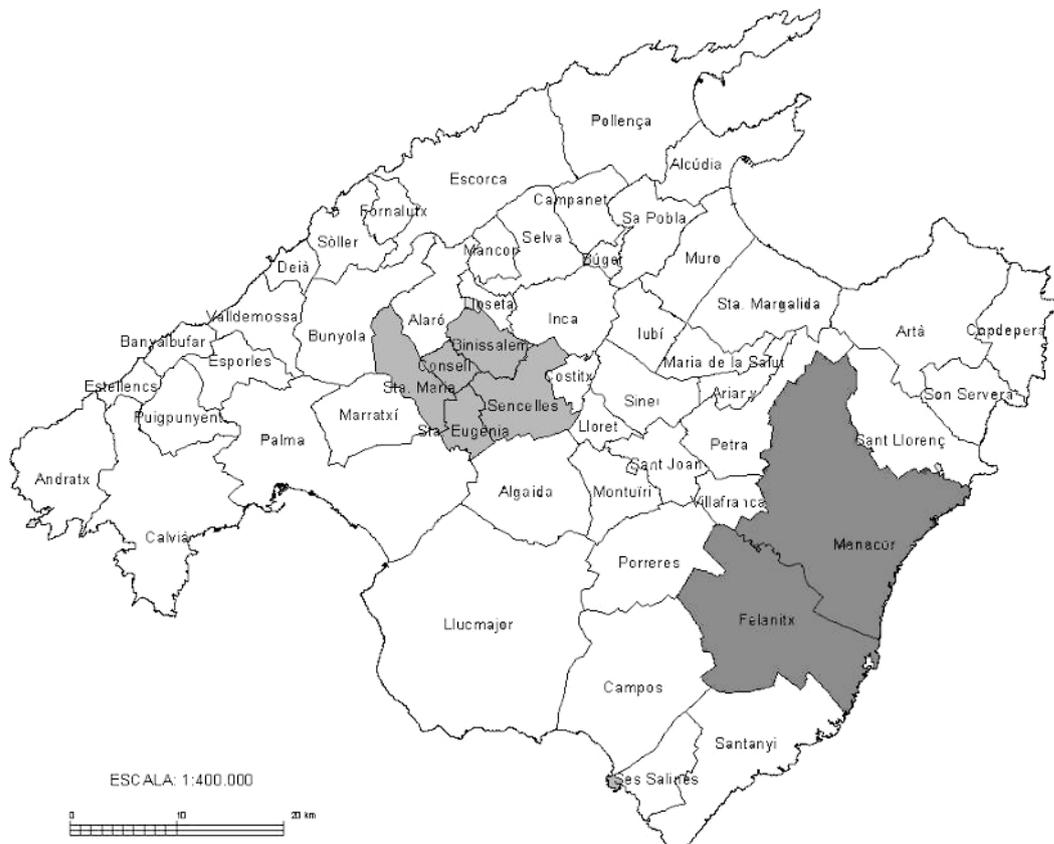


Figure 1. Map of Majorca highlighting municipalities in Binissalem-Mallorca (in the center of the island) and Pla i Llevant (on the east coast) appellations where vineyards included in clonal selection were chosen.

Table 1. Criteria for the selection of the candidate clones (from Carambula *et al.*, 2006)

Parameter	Manto Negro	Callet	Moll
100 berries weight (g)		100-230	
Yield per vine (g)	> 1,000		
Titrateable acidity (g L ⁻¹)	> 2.5		> 4
Sugar content (°Brix)	> 22	> 22	> 20
Total polyphenols index (A _{280nm})	> 25	> 20	
Total anthocyanins (mg L ⁻¹)	> 160		

and 2% w/v PVP, by using a mortar and pestle. The tissue to buffer ratio was 1:1 (g mL⁻¹). Crude extracts were put in 1.5 mL Eppendorf tubes and stored at -20°C. Samples were screened following the protocols described by Sánchez-Vizcaino and Cambra (1981). The double antibody sandwich (DAS) ELISA was carried out in the laboratories of the Virology Group of the «Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario» (IMIDA, Murcia, Spain), which is officially recognised by the «Ministerio de Medio Ambiente y Medio Rural y Marino», to perform sanitary tests for certification of grapevine planting material coming from every autonomous region of Spain. The antibodies used were purchased from Bioreba (Reinach, Switzerland). Crude extracts from three healthy plants were used as negative controls. The substrate used was p-nitro-phenyl phosphate at 1 mg mL⁻¹ in 10% v/v diethanolamine water solution (with 0.2 % w/v of sodium azide). Readings were performed at A_{405nm}. Samples were considered positive when readings were more than double of the average of controls (above 0.16 and 0.6, after 2 and 24 hours, respectively). Absorbance values between 0.13 and 0.16 and/or 0.4 and 0.6, respectively, were considered doubtful and tests were repeated.

For GLRaV-1, GLRaV-2 and GLRaV-3, the same protocol was followed. Samplings were only performed

in petioles in October. Negative tests by ELISA were readily repeated in the same year for further confirmation.

In 2005 all plants were also observed for symptoms of the viruses assayed and other possible infections.

Statistical analysis

Contingency table analyses were performed in order to study the relations among virus infection, appellation and variety. The values of χ^2 obtained were corrected by the factor of Yates (Little and Hills, 1981).

Results

The survey of infection incidence for the viruses studied suggested an unequal distribution between the two appellations and the three varieties assessed, and indicated that multiple infections are common.

To simplify the interpretation of the results, GLRaV-1, -2 and -3 were grouped as single pathogen (GLRaVs), being all of them associated to the same disease (leafroll complex). In the three varieties studied, GLRaVs infection was the most common (Table 2), showing a bigger incidence in Binissalem-Mallorca ($p < 0.05$, Table 2) where it was close to 100%, with just one Callet and one Manto Negro vines testing negative for all three viruses assayed. The incidence of GLRaVs in Moll vines in this appellation was also considerable (85%). GFLV infection was the second most common; its incidence with respect to Callet vines was greater in Pla i Llevant than in Binissalem-Mallorca ($p > 0.05$, Table 2). GFkV infection was the least common, especially in Pla i Llevant for Moll and Callet (Table 2). In addition, this virus seems to be very unequally present among varieties (Table 5).

Table 2. Incidence of each virus per variety and appellation (in %)

Virus	Manto Negro			Callet			Moll			Global total		
	Bi	PL	Total	Bi	PL	Total	Bi	PL	Total	Bi	PL	Total
GFLV	63 ^a	41 ^a	54 ¹	42 ^a	58 ^a	52	47 ^a	46 ^a	46	50 ^a	50 ^a	50
GLRaVs	96 ^a	41 ^b	71	97 ^a	67 ^b	78	85 ^a	50 ^b	69	93 ^a	56 ^b	73
GFkV	46 ^a	36 ^a	42	45 ^a	23 ^b	35	32 ^a	7 ^a	21	44 ^a	25 ^b	33

¹ Percentage of plants infected by each virus according to the ELISA. Letters a and b denote if differences between observed and expected data are significant or not significant at contingency table (presence/absence of each virus versus appellation, independence between variables considered as null hypothesis, $p < 0.05$). Bi: Binissalem-Mallorca appellation. PL: Pla i Llevant appellation. GFLV: *Grapevine fanleaf virus*. GLRaVs: at least one of grapevine leafroll-associated viruses. GFkV: *Grapevine fleck virus*.

Table 3. Simple and multiple virus incidence per variety and appellation (in %)

Virus infection status	Manto Negro			Callet			Moll			Global total
	Bi	PL	Total	Bi	PL	Total	Bi	PL	Total	
Certiﬁable virus free	0.0	13.6	6.3	3.3	13.5	9.6	5.9	17.9	11.3	9.4
GFLV	3.8	18.2	10.4 ¹	0.0	13.5	8.4	8.8	25.0	16.1	11.4
GLRaVs	11.5	22.8	16.6	12.9	17.3	15.7	26.5	35.7	30.6	20.7
GfKv	0.0	18.2	8.3	0.0	3.8	2.4	0.0	0.0	0.0	3.1
GFLV+GfKv	0.0	9.1	4.2	0.0	1.9	1.2	0.0	7.1	3.2	2.6
GFLV+GLRaVs	38.5	9.1	25.0 ²	29.0	32.7	31.3	26.5	14.3	21.0	26.4
GfKv+GLRaVs	23.1	4.5	14.6	41.9	7.7	20.5	23.5	0.0	12.9	16.6
GFLV+GfKv+ GLRaVs	23.1	4.5	14.6	12.9	9.6	10.8	8.8	0.0	4.8	9.8

Abbreviations: See Table 2. ¹ Percentage of plants infected by only GFLV according to the Enzyme-Linked Immuno-Sorbent Assay. ² Percentage of plants infected by both GFLV and at least one of GLRaVs assayed.

Viral symptoms were observed in most vines with positive ELISA tests. Visual detection was 88.2% and 93.8% for leafroll (associated with GLRaVs), and 76.9% and 81.0% for fanleaf (caused by GFLV) in Manto Negro and Callet, respectively. No symptoms were observed in Moll vines. Symptoms were never observed in vines displaying negative ELISA tests. GfKv infection and Rugose Wood complex were not visually detectable in any variety.

The incidence of multiple virus infections was also studied (Table 3). The percentage of plants showing multiple infections was 58.4%, 63.8%, 42.6% for Manto Negro, Callet and Moll, respectively. The most common multiple infection in each variety was GFLV+GLRaVs, with all three varieties showing an incidence of more than 20%. The triple infection was observed in both Callet and Manto Negro in more than 10% of vines, while being lower in Moll (4.9%, Table 3).

The incidence of GLRaV-1, GLRaV-2 and GLRaV-3 infections in GLRaVs-infected vines is also presented separately (Table 4). GLRaV-3 was the predominant

virus in Moll and Callet (97.7% and 90.7%, respectively), being also significant in Manto Negro (50.0%). Single infection by GLRaV-3 was the most common in Moll and Callet, being 44.6% and 32.6%, respectively. In Manto Negro, the most common virus infection was by GLRaV-1 (79.4%); single infection by GLRaV-1 was 50% in this variety. Grapevine Leafroll associated Virus 2 was never present alone. Triple GLRaVs infection was relevant in each variety, while GLRaV-1+GLRaV-3 was the most important GLRaVs double infection, especially in Moll where it accounted for more than 40% (Table 4). Both GLRaV-1 and GLRaV-3 seem to be linked to the grapevine variety, showing notable different incidences in Manto Negro with respect to Callet and Moll (Table 5).

Regarding to candidate clones chosen for the evaluation in homologation fields, only one vine of Manto Negro displayed the status of «certifiable virus-free». No more «certifiable virus-free» vines could be chosen as candidate clones because they did not meet the minimum agronomic requirements (Table 1). The ELISA performed in replicates of candidate clones confirmed

Table 4. Incidence of different Grapevine leafroll associated viruses in GLRaV-infected vines (in %)

Variety	GLRaV infection status							Total R ₁	Total R ₂	Total R ₃
	R ₁	R ₂	R ₃	R ₁ R ₂	R ₁ R ₃	R ₂ R ₃	R ₁ R ₂ R ₃			
Manto Negro	50.0 ¹	0.0	20.6	0.0 ²	2.9	0.0	26.5	79.4 ³	26.5	50.0
Callet	6.2	0.0	44.6	3.1	15.4	3.1	27.6	52.3	33.8	90.7
Moll	2.3	0.0	32.6	0.0	41.9	7.0	16.3	60.5	23.3	97.7

R₁: Grapevine leafroll associated virus 1 (GLRaV-1); R₂: Grapevine leafroll associated virus 2 (GLRaV-2); R₃: Grapevine leafroll associated virus 3 (GLRaV-3). ¹ Percentage of GLRaV-infected vines displaying infection by only GLRaV-1. ² Percentage of GLRaV-infected vines displaying infection by GLRaV-1 and GLRaV-2. ³ Percentage of GLRaV-infected vines displaying infection by GLRaV-1.

Table 5. χ^2 values calculated by means of a contingency table for each virus and couple of varieties, considering the independence between variables as null hypothesis

Virus (presence/ absence)	Variety comparison		
	Manto Negro × Callet	Manto Negro × Moll	Callet × Moll
GFLV	0.006 n.s.	0.833 n.s.	0.667 n.s.
GFkV	0.166 n.s.	3.045*	6.945**
GLRaV-1	3.166*	1.684 n.s.	0.058 n.s.
GLRaV-3	14.454**	10.105**	0.063 n.s.

Abbreviations: See Table 2. n.s.: not significant. **: significant differences between observed and expected data at $p < 0.1$ and $p < 0.05$, respectively.

the results obtained for mother plants with more than 96% coincidence with respect to the virus status infection (data not shown). Some cases of reinfection by GFkV were found in the homologation field of Binissalem. No virus infection was found in any replicates of the «certifiable virus-free» clone of Manto Negro.

Discussion

Sanitary tests and viruses assayed

Despite the advances in molecular diagnostic of viruses (Gambino and Gribaudo, 2006), the ELISA method may still be considered the most suitable for routine large scale testing of field samples, although biological tests (indexing) cannot be dismissed yet to achieve high levels of confidence (Rowhani *et al.*, 2005). Besides the viruses listed by the Council Directive 68/193/EEC, GLRaV-2 was also tested because of its importance in Spanish viticulture (Padilla *et al.*, 2007). Other GLRaVs (GLRaV-4, GLRaV-5, GLRaV-6, GLRaV-7, GLRaV-8, GLRaV-9) were not assayed because they are uncommon in Spain (Padilla *et al.*, 2007). Serological tests for the detection of the viruses linked to RW were not performed for two reasons: a) the absence of visual symptoms and b) the fact that in Spain RW complex seems to represent a severe problem in table grape varieties much more than in wine grape varieties (Padilla *et al.*, 2007). In spite of the spreading of his nematode vector (*Xiphinema diversicaudatum*) in Spain, the test for ArMV was not performed since it was found in grapevine only in very rare occasions in Spanish areas with Atlantic-like climate (Aballeira *et al.*, 2009).

Virus incidence

It is well documented that viral infections reduce productive life in grapevine plants (Andret-Link *et al.*, 2004; Padilla *et al.*, 2007). The observation that Callet and Manto Negro vineyards more than 30 years old presented very poor agronomic conditions and high plant losses was the first indication of a severe incidence of viral infection in Majorcan viticulture.

The high incidence of virus infections in small populations of local varieties complicates their clonal selection (Salazar, 1985; Borgo *et al.*, 2000; Poljuha *et al.*, 2004; Spinthiropoulou *et al.*, 2004; Materazzi *et al.*, 2006; Zduniç *et al.*, 2007). In Majorca two main factors may have lead to such a virus incidence. First, in the Balearic Islands there are no grapevine nurseries and no certified grape propagation material is available from any of the three varieties studied. Therefore the majority of the vineyards of Manto Negro, Callet and Moll were established from buds of unknown sanitary status. Second, historic records attest that for over one-hundred years, the preselected vineyards have been under grape cultivation, without rotation with other crops. At the moment there is evidence of the presence of the nematode vector of GFLV (*Xiphinema index*) in Mallorca (Talavera, 2005), but no study showing its spreading is available. Nevertheless, under these conditions, proliferation of *Xiphinema index* is likely to be high (Hanna *et al.*, 2008). Extensive information about mealybugs is lacking for Majorca. These phytophagous insect vectors do not represent a direct problem for the local viticulture industry, and therefore control measures are usually not taken. However there is evidence of *Planococcus citri* presence in vineyards of the Balearic Islands (Conselleria d'Agricultura i Pesca, Govern de Les Illes Balears, personal communication), and this insect is a well-known vector of GLRaV-3 (Cabaleiro *et al.*, 2008). The combination of a long history of viticulture, the presence of insect vectors, and the lack of sanitary stocks have possibly contributed to a high incidence of viral infection.

Regarding the different distribution of the viruses, at the moment it is very complicated to justify why a given virus is more present in a given variety or in a given appellation since there is no study about the origin (temporal and spatial) of these varieties and their relations with the viruses studied, as well as there is not any evidence on differential cultural practices or vector spreading which could have led to a differential virus spreading among appellations.

Obtaining standard material

The clonal selection process is now decades old. Despite an established history, it remains unclear whether genetic improvement or sanitary aspects are the most important in improving varietal performance (Walter and Martelli, 1997; Mannini, 2003). Clonal selection should take into consideration two factors: first, the tendency of modern viticulture to establish polyclonal vineyards (Martínez de Toda, 2000), and, second, the potential for clonal selection to reduce the natural genetic variability of a variety (Mannini, 2000; Martínez de Toda, 2000). The level of intravarietal variability is higher in varieties which originated from more than one close related seedlings (poly-clonal origin, Sensi *et al.*, 1996; Silvestroni *et al.*, 1997), and depends on the cycles of propagation, the spread to different locations and the intensity of cultivation by growers (Regner *et al.*, 2006).

For varieties with a wide distribution, it is quite easy to find several clone selections, and globalization has facilitated the propagation and interchange of stocks between countries. For example in Australia certified clones of Pinot noir have been imported from Canada, California, Switzerland, Germany and France (Nicholas, 2006). In Spain, certified clones of Tempranillo coming from different autonomous regions are available (Rubio and Yuste, 2004). Therefore, when a new clonal selection is performed the diversity of available material should be considered. If a considerable number of clone selections are already available, obtaining clones with sanitary guarantees is a primary goal (Grenan *et al.*, 2000).

However, where no certified clones are available, sanitary goals must be balanced against the goals of preserving diversity. Thus, clonal selection within local varieties is challenging since the goal of preserving intravarietal heterogeneity may be in conflict with sanitary aims (Blanco *et al.*, 2004). Manto Negro, Callet and Moll are cultivated only in the Balearic Islands, so one of the goals of clonal selection should be the achievement of the highest possible number of clones with different agronomic features. The degraded status of the autochthonous vineyards suggests that the process is imperative in order to preserve these varieties. In the case of Majorca, the low number of candidate clones free of virus infection adds financial and temporal costs to the process of developing certified clones, a process that is already expensive (Schöffling, 1997). Performing sanitation is not always a guarantee of success, especially in the case of multiple infections

(Gribaudo *et al.*, 2006). Thus, in our opinion, a good option could be evaluating candidate clones in homology fields without previous sanitation. This approach may lead to the identification of remarkable standard category clones and their availability for producers in a shorter period of time, separately from sanitation efforts. In situations where sanitary goals must be compromised, we argue that traceability should be superimposed on standard category «clones» through the assignment of identification numbers to each mother plant.

The large number of indigenous varieties in use at the regional and provincial levels in Europe (Mannini, 2004) suggests that clonal selection for the improvement of vine performance may need to balance the goals of genetic improvement and sanitation. For autochthonous varieties it is strongly recommended to combine clonal selection with the creation of a genetic reservoir in order to protect the varietal diversity (Jacquet *et al.*, 2000; Jung and Maul, 2004). Although such a reservoir may be established prior to the identification of virus free clones, it does not deny the importance of sanitation.

Our efforts have identified a «certifiable virus-free» clone of Manto Negro and a number of clones for genetic conservation and, possibly, for using as standard category «clones» in a short time. Considering the potential use of these varieties elsewhere and the additional sanitary requirements introduced by the Commission Directive 2005/43/EC (OJ, 2005) for standard material, sanitation of other candidate clones will be a necessary step to improve the performance of the three varieties.

In order to avoid genetic erosion in local grapevine varieties, clonal selection should provide a reasonable number of distinguishable clones. Where the incidence of virus infection is normally very high for autochthonous varieties, as the case of Majorca, the use of standard category «clones» offers a transitional solution, but the creation of a traceability system is strongly advised. Finally, the achievement of certified clones through sanitation should be the ultimate goal.

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Annex. Distribution of the vines evaluated between the vineyards included in the study and their virus status infections

Vineyard	Certifiable virus free plants	GFLV plants ¹	GFkV plants	GLRaVs plants	GFLV+ GFkV plants ²	GFLV+ GLRaVs plants	GLRaVs+ GFkV plants	GFLV+ GLRaVs+ GFkV plants ³	Total plants
<i>Callet</i>									
Bi 01				1		2			3
Bi 12						3	2	1	6
Bi 14	1					2	3		6
Bi 15						2	2	2	6
Bi 20				1			1		2
Bi 25				2			5		7
Bi 30								1	1
PL 02		2			1			1	4
PL 03			1						1
PL 09	3	1		5		3	1		13
PL 10	2	1	1				3	4	11
PL 23						2			2
PL 25		2				2			4
PL 28				1		2			3
PL 36		1		1		1			3

Annex (cont.). Distribution of the vines evaluated amongst the vineyards included in the study and their virus status infections

Vineyard	Certifiable virus free plants	GFLV plants ¹	GFkV plants	GLRaVs plants	GFLV+ GFkV plants ²	GFLV+ GLRaVs plants	GLRaVs+ GFkV plants	GFLV+ GLRaVs+ GFkV plants ³	Total plants
PL 49	1								1
PL 53						2			2
PL 60				1		2			3
PL 61						3			3
PL 70	1			1					2
Total	8	7	2	13	1	26	17	9	83
<i>Manto Negro</i>									
Bi 04				1				1	2
Bi 10						1		1	2
Bi 12						2	3		5
Bi 14		1				3			4
Bi 15							2	1	3
Bi 16						1		1	2
Bi 20				1		1	1	1	4
Bi 30				1		2		1	4
PL 03	1	1							2
PL 15			2		1		1		4
PL 23				1	1				2
PL 29	1		2					1	4
PL 35	1			5					6
PL 52		3				2			5
Total	3	5	4	8	2	12	7	7	48
<i>Moll</i>									
Bi 15						4	6	2	12
Bi 17				8		2	2	1	13
Bi 25	2	3		1		3			9
PL 03	1					1			2
PL 08				4			2		6
PL 31	1	2				1			4
PL 46				1					1
PL 51	1	2							3
PL 52		1		2					3
PL 53				2					2
PL 62	2	3			2				6
Total	7	11	0	18	2	11	10	3	62

GFLV: *Grapevine fanleaf virus*. GFkV: *Grapevine fleck virus*. GLRaVs: at least one of grapevine leafroll associated viruses. Bi: Binissalem. PL: Pla i Llevant. ¹ Plants infected by only GFLV. ² Plants infected by GFLV and GFkV. ³ Plants infected by GFLV, GFkV and GLRaVs.