IMPLICATION OF SECONDARY VIRAL INFECTIONS ON GRAFTING SUCCESS RATED IN NURSERIES

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Abstract:

Context and purpose of the study

Grapevine grafting is a complex process that since the establishment of phylloxera has become mandatory for grapevine. Grafting success in grapevine nurseries considerably varies among years and batches with most variety/rootstock combinations reach a high success rate (between 75% and 90%), but some combinations show lower success rates of around 40-50%. The causes of this variation are unknown, although biotic stresses like those caused by some viral infections have been demonstrated to affect the process. European certification schemes for the vegetative propagation of the vine include five major viruses (Arabis mosaic virus, Grapevine Fanleaf Virus, Grapevine Fleck Virus, and Grapevine-associated Leafroll Virus 1 and 3). However, other viral infections are ubiquitous in plant material and may affect the grafting process. The present study aimed to assess the presence of these other 'secondary viruses', and their influence on the grafting process.

Material and methods

Plant material was collected from commercial mother plant fields located in Larraga (Navarra, Spain). The presence of Grapevine virus A (GVA), Grapevine virus B (GVB), Grapevine-associated Leafroll Virus 2 Pinot noir (GLRaV-2 PN), and Rupestris Stem Pitting-associated Virus (RSPaV) was assessed by RT-PCR to determine the most common secondary viruses in the plant material. Mother fields for 7 scion (Tempranillo, Chardonnay, Airen, Macabeo, Sauvignon blanc, Garnacha, and Mazuelo) and 5 rootstocks (110 Richter, Rafa García 8 (RG8), Rafa García 9 (RG9), Millardet et Grasset 41 B, and 140 Ruggeri) were tested. Considering the prevalence observed for the different viruses, the influence of the virus on grafting success was tested for Tempranillo grafted onto 110R, since for this material it was possible to use combinations of virus-free and virus-infected scion and rootstock wood. The relation between the viral presence and the success rate was tested by Pearson chi-square.

Results

RSPaV had the highest incidence (55%), and a difference was observed between varieties and rootstocks (85% vs. 25%). GLRaV-2 was detected in 1.3% of the samples and GVA and GVB were not detected. Three sanitary conditions were established based on the detected infections: I) virus-free, II) RSPaV, and III) RSPaV + GLRaV-2. Tempranillo samples had conditions II and III, while 110R had conditions I and II. A minimum of 24 plants were grafted for each combination (variety/rootstock): RSPaV/virus-free, RSPaV/RSPaV, GLRaV2+RSPaV/virus-free, and GLRaV2+RSPaV/RSPaV. The success rate for the RSPaV/virus-free combination was 72%, for RSPaV/RSPaV combination was 54%, GLRaV2+RSPaV/virus-free was 36%, and for GLRaV2+RSPaV/RSPaV was 25%. The Chi-square test determined a significant relationship between the success rate and the sanitary conditions of the graft, which statistical results suggested was determined by the sanitary condition of the variety and not in the rootstock. The residual analysis of the Chi-square test, which related expected success with sanitary status, had the most positive association for RSPaV/virus-free graft combination while the most negative was for GLRaV2+ RSPaV / RSPaV. The results of the study point to widespread secondary viruses such as GLRaV-2 and RSPaV that could be involved in graft incompatibility.

Keywords: Grapevine, viruses, graft success rate.

1. Introduction

The outbreak of phylloxera (*Daktulosphaira vitifoliae*) in Europe in the second half of the 19th century led to the uncontrolled propagation of the disease and the grafting of grapevine material onto phylloxera-tolerant rootstocks. This situation provided ideal conditions for mixing and spreading viruses from one cultivar to another (Krake et al., 1999). And since the early 1960s, nearly 70 different virus species and virus-like agents have been identified in grapevine species worldwide (Rowhani et al., 2017).

Viral diseases' complexity lies in the large number of viruses that can infect grapevine plants, which can cause multiple infections(Armijo et al., 2016). Viral infections in grapevine plants cause diverse biotic stresses, affecting vegetative organs (v.gr. inducing leaf deformations and alterations in leaf colour), producing irregular ripening or, in some cases, causing graft rejection (Martelli, 2017). Furthermore, multiple infections of the grapevine by two or more viruses are a common phenomenon, often leading to increased symptoms (Kominek et al., 2009).

Of these diseases, several viruses have been linked to graft incompatibility as being associated with abnormal development of conductive tissues. A strong association between GLRaV-2 and the Kober 5BB graft incompatibility condition was found in French and Italian grapevines (Bertazzon et al., 2010). Also grapevine rugose wood (GRW), in which aetiology are at least five different viruses involved, i.e. Grapevine virus A (GVA), Grapevine virus B (GVB), Grapevine virus C (GVC), Grapevine virus D (GVD), and Rupestris stem pitting associated virus-1 (RSPaV-1) (Meng et al., 1999), produces swellings and grooves-likes depressions in the wood under the bark at the grafting point, both in the variety and in the rootstock (Martelli, 1993).

Although the control of those viruses is not regarded in the plant material certification schemes (ECC, 1968; OEPP, 2008), any of these viruses are present in the plant propagation process carried out in nurseries, compromising the sanitary quality of the propagation material. The current success rate for most variety/rootstock combinations exceeds 75 %. However, some combinations show success rates of around 40-50% (Pisciotta et al., 2017). The causes of this variation are unknown, although previous research revealed that the presence of non-mandatory testing viruses or 'secondary' viruses was associated with incompatibility problems or low engraftment success rates (Martelli, 2017).

Information on the presence of graft incompatibility virus in Spanish vineyards is scarce. Therefore, the aim of this study is, firstly, to know the sanitary status of the plant material in terms of "secondary" viruses and then, to carry out nursery trials to assess the effect of these diseases on graft incompatibility.

2. Material and methods

This study was carried out in three differentiated phases: (i) collection of leaf grapevine samples, (ii) detection of viruses in grapevine samples, and (iii) nursery trial.

Collection of leaf grapevine samples - The samples were collected in August 2021 from Vitis Navarra nursery commercial fields located near Larraga in Navarra, Spain (42°34N, 1°50W). Rootstock samples were obtained from mother fields of 41 B Millardet et de Grasset, 110 Richter, 140 Ruggieri, RG8, and RG9 and scion samples were obtained from fields of cultivars Airén, Chardonnay, Garnacha, Macabeo, Mazuelo, Sauvignon Blanc, and Tempranillo. Leaf samples were randomly collected and stored at -80°C until further analysis. Diverse plant material was selected in order to obtain a general picture of viral incidence, while further grafting trials were performed with 110R and Tempranillo.

Detection of viruses in grapevine samples – Virus tests were run in the Public University of Navarre facilities. The material was tested for the presence of Grapevine virus A (GVA), Grapevine virus B (GVB), Grapevine-associated Leafroll Virus 2 Pinot noir (GLRaV-2 PN), and Rupestris Stem Pitting-associated Virus (RSPaV) by real time-PCR (RT-PCR). For that, 100 mg of plant material was ground to a fine powder. Total RNA isolation was performed using Spectrum Plant Total RNA Kit (Sigma-Aldrich, Oakville, ON, Canada) following manufacturer instructions with slight modifications: - 2% PVPP and 5µl β-Mercaptoethanol were added to the lysis buffer to avoid polyphenols and proteins, and - the elution step was repeated twice to increase RNA yield. 500ng of total RNA

was reverse-transcribed using the PrimeScript RT Reagent Kit (Takara Bio Inc., Shiga, Japan) following manufacturer instructions. Real-time amplification was carried out in an ABI StepOne Plus thermocycler (Applied Biosystems, Foster City, CA, USA). PCR mixture included 10ng of cDNA, 1x TB Green Premix Ex Taq II and 1x ROX reference dye from a kit (Takara Bio Inc., Shiga, Japan), 0.4µM forward and reverse primers (Thermo Fisher, Waltham, MA USA), (Takara Bio Inc., Shiga, Japan) in a final volume of 10µl. Amplification conditions were according by the published by the authors (Table 1).

Table 1. Sequences of the forward and reverse primers used for RT-PCR

Viral agent	Primer name	Sequence	Reference
Grapevine Leafroll-associated virus strain PN (GLRaV-2 PN)	GLRaV-2 198 f GLRaV-2 290 r	F: CATTATATTCTTCATGCCTCTCAGGAT R: GATGACAACTTCTGTCCGCTATAGC	(Osman et al., 2008)
Grapevine Virus A (GVA)	GVA-F GVA-R	F: GACAAATGGCACACTACG R: AAGCCTGACCTAGTCATCTTGG	(Poojari et al., 2016)
Grapevine Virus B (GVB)	GVBmu-91f1 GVBmu-202r1	F: CTAGGAGTGCGGCTAAACGAA R: CCATATCACAGGACGAGGTTAAGG	(Osman et al., 2013)
Rupestris Stem Pitting-associated Virus (RSPaV)	RSP13 RSP14	F: GATGAGGTCCAGTTGTTTCC R: ATCCAAAGGACCTTTTGACC	(Meng et al., 1999)

Nursery trial –Grafting tests were performed in the nursery Vitis Navarra (Larraga, Navarra, Spain). The grafting combination was *V. vinifera* cv Tempranillo VN100 for the scion onto 110 Richter rootstock. Three sanitary conditions were established based on the detected infections: I) virus-free, II) RSPaV, and III) RSPaV + GLRaV-2. Tempranillo samples had conditions II and III, while 110R had conditions I and II. A minimum of 24 plants were grafted per sanitary condition. Grafted plants were obtained following the Vitis Navarra nursery protocol. Dormant canes (scion and rootstock) were initially collected from the mother plants in winter. Then, the canes were kept in cold storage at 4 ºC and, disinfected until grafting. Following disbudding, the rootstock and scion were spliced together, in omega-cut grafting, ensuring that the vascular cambium of both was aligned. The partners were connected via callus formation at the graft union, during a process called callusing. Upon successful callusing, grapevines were planted directly in the nursery field for one growing season to allow the grafted plant to develop. At the end of the season, plants were uprooted and kept in cold storage. To evaluate the success rate per category, plants that did not fulfil the technical features of a successful grafted (no vegetative development, poor root development, lack of resistance to the “thumb test”, a manual test used to evaluate the mechanical the strength of the union) were considered unsuccessful.

Statistical analysis – The chi-square test (χ 2) was performed to test for significant differences between success rate and health conditions. A χ 2 test was considered significant at the 5% level if the p-value was less than or equal to 0.05. Data analysis was performed using R Studio version 3.6.1 statistical software (RStudio Team, 2020).

3. Results and discussion

3.1. *All the Vitis vinifera cultivars tested were positive for the Rupestris Stem Pitting-associated Virus (RSPaV).*

From the 80 randomly selected samples, 44 (55%) tested positive for RSPaV, and 1 (1,3%) was positive for GLRaV-2 PN while GVA and GVB were not detected (Table 2). Only one sample tested positive for mixed infection, RSPaV + GLRaV-2. The survey carried out in Navarra (Spain), demonstrated that RSPaV is ubiquitous in grapevine plant material while the GLRaV-2PN strain has a very low incidence and can be considered as rare. In accordance with the RSPaV prevalence study carried out in 1980-1981 by Goheen, 1989, the virus was already identified in 66% of the 70 selected vineyards in France, in 42% of the 53 vineyards selected in Germany and in 67% of the 33 vineyards selected in Australia. RSPaV is probably the most widespread virus infecting Vitis spp. in all viticultural areas of the world (Mannini and Digiaro, 2017). Also, indexing records follow in California over 23 years indicated that 30.5% of the 6,482 grapevine selections introduced from around the world were infected with RSP (Golino and Butler, 1990). All those records, raise the need for a better knowledge of the pathological aspects induced by RSPaV as well as the development of management strategies for its control might be considered.

Furthermore, a rate difference was observed between varieties and rootstocks (85% vs. 25%). The health condition of the rootstocks was notably better, with only GRSPaV having an incidence of 25%. According to the results of Meng et al., (2006), GRSPAV, due to its geographical origin, may have co-existed with *V. riparia* and *V. rupestris* for a long time. Since *V. riparia* and *V. rupestris* vines or their hybrids have been commonly used as rootstocks, their " susceptibility " to the virus may be higher than that of *V. vinifera*.

3.2. *The grafting success rate was affected by the sanitary condition.*

The diseases are generally found on *Vitis vinifera L*. cultivars in a latent state and symptoms do not usually appear until the buds of diseased vines are grafted onto certain American rootstocks, at which point they adversely affect growth and yield (Credi, 1997).

The number of surviving grafted vines was in line with the normal values obtained in the nursery, around 70-80 %, for the II/I combination (Table 3). However, the other combinations had a lower success rate, between 54% and 25%. The number of grafted plants when the variety was infected by GRSPaV was higher than when both viruses (RSPaV+GLRaV-2) were present in the variety. The success rate decreased by half, from 72% to 36% and from 54% to 25%. Our trial confirms the results obtained by (Greif et al., 1995), which associated graft incompatibility with the presence of grapevine leafroll-associated virus 2 (GLRaV-2). Besides, we found that with the rootstock without RSPaV (II/I), grafting success was higher (72%) than with the rootstock with RSPaV (II/II). This is in line with research showing the impact of the virus on graft incompatibility (Golino, 1993).

The influence of each virus on the grafting success rate was also investigated for both variety and rootstock. Overall, the number of surviving vines was most affected by the virus variant of the variety and not by the rootstock, with a statistically significant interaction ($\chi^2_{(df=3)} = 16.569$ $P = 0.0008666$). The residual analysis of the Chi-square test, which related expected success to sanitary conditions, had the most positive association for the RSPaV/virus-free graft combination. The most negative association was for GLRaV2+ RSPaV / RSPaV.

4. Conclusions

Our results conclude that the virus RSPaV was widespread within the commercial plant material tested, as has been previously found in other regions around the world. Furthermore, GLRaV-2 strain PN was rare but appeared in a low rate. The presence of those viruses influenced the success rate of the grafting process. In special, when GLRaV-2 PN and RSPaV were combined a synergetic effect was induced.

5. Acknowledgments

This research received funding from the European Regional Development Fund through the FEDER Operational Program 2014-2020 of Navarra. Ana Villa-Llop is the beneficiary of an Industrial pre-doctoral contract of the Government of Navarra (Ref. 283E/2020). The authors thank Vitis Navarra nursery, especially Rafael García and Javier Eraso.

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Table 2: Number of positive samples detected for each grapevine virus analyzed by variety and rootstock.

	Nb samples	Non-confirm	Virus Free	GLRaV2	GVA	GVB	RSPaV	% of virus infection
Varieties	40	4 (10%)	2 (5%)	0 (0%)	0 (0%)	0 (0%)	34 (85%)	85%
Rootstocks	40	9 (23%)	20 (50%)	1 (3%)	0 (0%)	0 (0%)	10 (25%)	28%
% virus incidence		16%	28%	1%	0%	0%	55%	

$X^2_{(df=2)} =$ 29.205 $P = 4.552e\text{-}07$

Table 3: Success rate (%) for each sanitary condition combination.

Sanitary Condition - Variety / Rootstock	Id.	Success Rate (%)
RSPaV / Free	II / I	72%
RSPaV / RSPaV	II / II	54%
RSPaV-GLRaV2 / Free	III / I	36%
RSPaV-GLRaV2 / RSP	III / II	25%

$X^2_{(df=3)} = 16.569$ $P = 0.0008666$