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Evaluation of criteria to assist the selection of good quality grafted grapevines prior to their commercialisation

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ABSTRACT

The production of grafted grapevine plant material is a complex process with many steps running over at least one year, from grafting to final sorting in nurseries. To reach the market in France, grafted grapevines must meet three criteria by law: resistance to a manual graft union test (or thumb test), a minimum number of three roots and a woody, lignified stem which has grown from the bud of the scion wood of at least 2 cm long. This study aimed to evaluate the possibility of using visual criteria to select good quality grafted grapevines, without the need to do the thumb test because the thumb test is manual and therefore very subjective; the test depends on the strength applied by the person who realises it. This study was done on 22 scion/rootstock combinations with different degrees of grafting success, i.e., producing different proportions of marketable plants after one year in the nursery. The three legal criteria currently used to select marketable grafted grapevines in France as well as other external and measurable criteria such as the length of lignified stem and diameter, the number of thin and thick roots, and rootstock wood diameter were measured on the 22 scion/rootstock combinations. Variation in the values for these different criteria was observed and correlations between the criteria and the number of marketable plants were studied. This data was then analysed to determine which visible criteria contribute most to identifying marketable grafts. The percentage of marketable grafts was most strongly correlated with the thumb test and positively correlated with the length of the lignified stem. The variables with the highest predictive effect for identifying marketable plants (other than the thumb test) were the number of large roots and the length of the lignified stem. The possibility of using visible criteria to screen for good quality grafted plants is discussed, but no single, or combination of criteria, was sufficiently strongly correlated with the percentage of marketable plants to replace the thumb test.

KEYWORDS: graft union formation, nursery, grafting success, scion/rootstock, growth, plant quality, quality criteria

INTRODUCTION

Grafting is an ancient technique that is still widespread in horticulture for numerous woody and herbaceous plants (Mudge *et al.*, 2009). Until the end of the 19th century, the European grapevine, *Vitis vinifera*, was grown un-grafted on its own roots. The accidental introduction, via American *Vitis* spp., of the soil-dwelling aphid pest Phylloxera (*Daktulosphaira vitifolia*) induced a major crisis by devastating European vineyards (Pouget, 2015). Grafting *V. vinifera* varieties onto roots of American *Vitis* spp., tolerant to this pest, was found to be the only viable solution (Pouget, 2015). Grafting is currently used in almost all wine-growing countries. Grafting provides not only resistance to soil-borne pests but can also improve adaptability to the various soils and environment, and as such, is also used to improve fruit yield and quality (Ollat *et al.*, 2016).

There is no precise definition of what a successfully grafted plant is; it could be defined as the establishment of a successful graft union and extended survival and functioning of the resulting grafted plant. The processes involved in forming the graft union and the causes of poor grafting success remain poorly understood (Gautier *et al.*, 2019; Goldschmidt, 2014). The reasons for poor grafting success are not always known but can include genetic incompatibility pathogens and can be related to technical problems such as poor-quality plant material, climatic factors or poor workmanship.

Grafting success has been well-studied in fruit trees and horticultural and vegetable crops (reviewed by Pina *et al.* (2017)); fewer studies have been on grapevine. Some cases of graft incompatibilities have been described concerning grapevine, which can be genetic incompatibility or linked to certain pathogens. Genetic incompatibility is observed when certain scion/rootstock combinations are grafted together, and sometimes with specific clones for each partner (reviewed by D'Khili (1994)), and it could be the cause of heterogeneous development of certain scion/rootstock combinations (Todic *et al.*, 2005). Some graft incompatibilities are also induced by viral infection with specific virus-host combinations, such as some cultivars infected with grapevine leafroll-associated virus 2 (GLRaV-2) show incompatibility symptoms when grafted onto the rootstock Kober 5BB (Rowhani *et al.*, 2017).

Even in scion/rootstock combinations which normally graft well, the growth and development of grafts can vary, which suggests that there are differences in terms of "quality" in grafted plants. There is no clear definition of what constitutes a high-quality grafted grapevine plant; for Nicholas *et al.* (1992), it is defined as high health status, correct identity (varieties and clones), uniformity, being free from certain pests and handled in a manner that preserves their physiological competence. Waite *et al.* (2015) suggested that high quality grafted grapevines must also be undamaged, free of physical defects and present fully healed graft unions. To be sold in France, grafted grapevines must meet a certain number of regulatory criteria. Each plant must have at least three well-developed and regularly distributed roots, have a sufficient, regular and solid graft union, and have a minimum of 2 cm of a lignified stem that has developed from the bud that was present on the scion wood when grafted (Giry and Valade, 2006). The graft union solidity is evaluated manually by a test named the "thumb test": the operator presses on the graft union to check that it does not break easily. Among these criteria, the one concerning the graft union solidity is the most subjective and can vary greatly between different people and over time.

The objectives of this study were (i) to determine how the values of various grafted plant quality criteria vary across 22 different scion/rootstock combinations, which experience shows produce very different proportions of marketable grafts, (ii) to determine the relationships between the different criteria and finally (iii) to test whether the very subjective thumb test could be replaced by other visual criteria to select grafted grapevines for commercialisation.

MATERIALS AND METHODS

1. Plant Material

Twenty-two different scion/rootstock combinations were bench omega grafted in March 2019, comprising nine *V. vinifera* scion varieties grafted onto five different rootstocks (Table 1). They were selected to represent a range of behaviour concerning typical grafting success rates according to the experience of nursery workers (P. Bloy and O. Yobregat, IFV, personal communication). The same material was used in our previous study on the identification of metabolite markers of grafting success rate (Loupit *et al.*, 2022). Some scion clones known to be infected with GLRaV-2 (indicated by *) were used, namely, Grenache 363*, Sauvignon 316*, Macabeu 789*, Cinsaut 67* and Bourboulenc 541*. Two to three hundred grafts per combination were grafted at the Wine and Vine French Institute (IFV, Le Grau-du-Roi, France); in total, 4858 grapevines were grafted for this study.

2. Grafting

Over-wintering woody canes of both rootstocks and scions were collected from mother grapevines planted in two vineyards in Gard (France). The scion material came from IFV (Espiguette, Le-Grau-du-Roi, France) and the rootstocks from some mother vines established in Saint Jean de Marvejol (Gard, France). All the mother vines are monitored every three years to check the absence of certain viruses: *Grapevine leafroll-associated virus 1* and *3* (GLRaV-1, -3), *Grapevine fanleaf virus* (GFLV) and *Arabis mosaic virus* (ArMV).

The process typically used locally to produce grafted plants was applied to all the scion/rootstock combinations of this study. Prior to grafting, buds were mechanically abraded with brushes to remove them from the rootstock canes; the rootstock canes were then cut into pieces 28 cm long and kept in crates made of micro-perforated black plastic. The scion canes were cut into one bud cuttings and were also kept in micro-perforated plastic bags. Both were immediately

TABLE 1. The scion/rootstock combinations used in this study.

Abbreviation	Scion Genotype	Rootstock Genotype
Bourboulenc 541*/5BB 259	*V. vinifera* cv. Bourboulenc clone 541*	*V. berlandieri* × *V. riparia* cv. Kober 5 BB (5BB) clone 259
Cinsaut 67*/5BB 259	*V. vinifera* cv. Cinsaut clone 67*	*V. berlandieri* × *V. riparia* cv. Kober 5 BB (5BB) clone 259
Grenache 136/5BB 259	*V. vinifera* cv. Grenache Noir clone 136	*V. berlandieri* × *V. riparia* cv. Kober 5 BB (5BB) clone 259
Grenache 363*/5BB 259	*V. vinifera* cv. Grenache Noir clone 363*	*V. berlandieri* × *V. riparia* cv. Kober 5 BB (5BB) clone 259
Macabeu 789*/5BB 259	*V. vinifera* cv. Macabeu clone 789*	*V. berlandieri* × *V. riparia* cv. Kober 5 BB (5BB) clone 259
Merlot 343/140 RU 265	*V. vinifera* cv. Merlot Noir clone 343	*V. berlandieri* × *V. rupestris* cv. 140 Ruggeri (140 Ru) clone 265
Merlot 343/5BB 259	*V. vinifera* cv. Merlot Noir clone 343	*V. berlandieri* × *V. riparia* cv. Kober 5 BB (5BB) clone 259
Merlot 343/RSB1 141	*V. vinifera* cv. Merlot Noir clone 343	*V. berlandieri* × *V. riparia* cv. Rességuier Sélection Birolleau 1 (RSB1) clone 141
Merlot 343/SO4 762	*V. vinifera* cv. Merlot Noir clone 343	*V. berlandieri* × *V. riparia* cv. Sélection Oppenheim 4 (SO4) clone 762
Négrette 581/140 RU 265	*V. vinifera* cv. Négrette clone 581	*V. berlandieri* × *V. ruprestris* cv. 140 Ruggeri (140 Ru) clone 265
Négrette 581/SO4 762	*V. vinifera* cv. Négrette clone 581	*V. berlandieri* × *V. riparia* cv. Sélection Oppenheim 4 (SO4) clone 762
Négrette 582/5BB 259	*V. vinifera* cv. Négrette clone 582	*V. berlandieri* × *V. riparia* cv. Kober 5 BB (5BB) clone 259
Négrette 582/RSB1 141	*V. vinifera* cv. Négrette clone 582	*V. berlandieri* × *V. riparia* cv. Rességuier Sélection Birolleau 1 (RSB1) clone 141
Pinot noir 374/110R 163	*V. vinifera* cv. Pinot Noir clone 374	*V. berlandieri* × *V. rupestris* cv. 110 Richter (110R) clone 163
Pinot noir 828/110R 163	*V. vinifera* cv. Pinot Noir clone 828	*V. berlandieri* × *V. rupestris* cv. 110 Richter (110R) clone 163
Pinot noir 828/5BB 259	*V. vinifera* cv. Pinot Noir clone 828	*V. berlandieri* × *V. riparia* cv. Kober 5 BB (5BB) clone 259
Sauvignon 316*/5BB 259	*V. vinifera* cv. Sauvignon Blanc clone 316*	*V. berlandieri* × *V. riparia* cv. Kober 5 BB (5BB) clone 259
Ugni blanc 478/SO4 762	*V. vinifera* cv. Ugni Blanc clone 478	*V. berlandieri* × *V. riparia* cv. Sélection Oppenheim 4 (SO4) clone 762
Ugni blanc 481/5BB 259	*V. vinifera* cv. Ugni Blanc clone 481	*V. berlandieri* × *V. riparia* cv. Kober 5 BB (5BB) clone 259
Ugni blanc 482/RSB1 141	*V. vinifera* cv. Ugni Blanc clone 482	*V. berlandieri* × *V. riparia* cv. Rességuier Sélection Birolleau 1 (RSB1) clone 141
Ugni blanc 483/140 RU 265	*V. vinifera* cv. Ugni Blanc clone 483	*V. berlandieri* × *V. rupestris* cv. 140 Ruggeri (140 Ru) clone 265
Ugni blanc 483/S04 762	*V. vinifera* cv. Ugni Blanc clone 483	*V. berlandieri* × *V. riparia* cv. Sélection Oppenheim 4 (SO4) clone 762

* Scion varieties infected with grapevine leafroll-associated virus 2.

stored in a dedicated cold chamber (4 °C) with a maintained humidity (85 to 90 %). They were rehydrated in tap water 1 d before the grafting, directly in the perforated plastic bags for the scions and taken out of the bags for rootstocks. Mechanical omega grafting was performed with commercial machines (Omega Star, Chauvin, France) on scion/rootstock pairs with approximately the same diameter on 28 March 2019 by an experienced technician. Grafts were immediately dipped in melted paraffin containing dichlorobenzoic acid (Staehler Rebwachs pro and Optiwax (20-80), Chauvin) then tightly arranged in plastic crates. The crates were left at ambient temperature for 7 d before being placed in the callusing room. For the callusing step, each crate was covered by a plastic sheet to maintain humidity and promote callusing. The temperature of the callusing room was gradually increased from 18 °C to 28 °C during 4 d. Four days later, water with 0.2 % dichlorobenzoic acid and copper sulphate (40 mg L^{-1}) was added to the crates (about 2 cm of water). During the warm callusing step, grafted plants were continuously kept under controlled conditions at relatively high temperatures (27–28 °C) and high humidity (> 80 %).

The callus formation was monitored each day visually for each crate. When callus at the graft interface was visible on most grafted grapevines in a crate, it was taken out from the stratification chamber. It took between 4 to 10 d depending on the scion/rootstock combination. The liquid in the crates was then changed to water with 0.008 % of dichlorobenzoic acid for 2 d to promote rooting. The duration of the stratification procedure varied depending on the scion/rootstock combination.

After the stratification process, the grafts were sorted to keep only the plants with a "well-developed" callus at the graft interface, which means the callus covers the entire circumference of the graft interface). (Supplementary Figure 1A). The grafted vines presenting a "well-developed" callus (Supplementary Figure 1A) were then placed at ambient temperature and recoated with melted wax (Rhizopon with indolebutyric acid) while the other grafts with "poorly-developed" callus (Supplementary Figure 1B) were discarded; this was done 19 April 2019. The grafts were then planted in a field nursery of sandy soil covered with a black plastic on 26 April 2019. Drip irrigation and fertilisation were maintained during all the spring and summer, depending on the shoot development and the season. The plants were lightly trimmed 5 times from July to September. At the end of the growing season (11 December 2020), grafts were uprooted with a digger that trimmed the roots at about a depth of 40 cm. The grafted grapevines were stored in wooden boxes in the cold chamber until they were sorted according to official commercial criteria and measured for other characteristics.

3. Phenotyping grafting success at each key point of the process

The presence of well-developed callus was determined after the stratification period when the grapevines were first sorted before being recoated in wax (Supplementary Figure 1A). The percentage of grafts with a well-developed callus was calculated in relation to the total number of grafted grapevines. Shoot growth was assessed visually in the nursery in the spring (on 17 May 2019) to calculate the percentage of plants that had started growing, i.e., grafted grapevines with a growing green shoot. The final grafting success rate was defined as the percentage of marketable grafts (as defined by the legal criteria in France) relative to the number of the total grafted grapevines after plants were sorted in December 2019.

4. Detailed phenotypic characterisation of 30 plants per scion/rootstock combination

For two combinations (Macabeu 789*/5BB and Bourboulenc 541*/5BB), the number of plants alive in the nursery before uprooting was less than 10. In these two cases, no phenotypic characterisation was done because of the very low number of plants produced; only the percentage of marketable plants was calculated (4 % and 0 %, respectively).

For the other 20 scion/rootstock combinations, 30 plants were selected randomly for phenotypic characterisation after uprooting (except in the case of Grenache 363*/5BB, in which only 28 plants survived). The following growth parameters were measured: length of lignification of the main stem of the shoot, number of small (< 2.5 mm) and large (≥ 2.5 mm) diameter roots, stem diameter of the 2nd internode of the shoot, diameter of the rootstock wood (just below the graft union), and homogeneity of the distribution of the roots around the base of the trunk. The root development is considered homogeneous when the root density is similar all around the base of the rootstock or non-homogenous if it was not (this was scored in a binary fashion as homogenous or not). The roots and rootstock wood diameters were measured with a sliding calliper. The lignified stem length of the shoot was assessed by category: 1 (less than 2 cm), 2 (2 to 10 cm), 3 (10 to 20 cm), 4 (20 to 30 cm) and 5 (over 30 cm). In category 1 (less than 2 cm), the grapevines are unmarketable in France. For all the plants, the same person counted the number of roots with a diameter over and under 2.5 mm (i.e., the number of large and small diameter roots), assessed the root system homogeneity and measured the length of the lignified shoot. A second person measured the wood rootstock diameter and the shoot diameter at the 2nd internode. Callus regularity was assessed (this was scored in a binary fashion as either regular or irregular callus development around the graft interface) and the solidity of the mechanical graft union was tested manually (thumb test) by a unique and experienced technician.

Finally, each grafted grapevine was assigned to either the Marketable or Unmarketable class depending on the regulatory criteria in France defined above.

5. Statistical analysis

Statistical analysis of the data and figures were done in R (version 3.6.1 (R Core Team, 2018)), RStudio (version 1.2.5019) using gplots, ggplot2 and FactoMineR packages, SigmaPlot (Systat Software, San Jose, CA) and

BioStatFlow (v.2.9.2 © INRA 2019). Significance differences are reported at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, 'ns' non-significant differences. The Knowledge Discovery by Accuracy Maximization Analysis (KODAMA) was used for the semi-supervised identification of the most correlated variables with the thumb test. The number of repetitions of the procedure and the number of cycles were 100. In this method, the classifier was the Partial Least Square-Discriminant Analysis (PLS-DA). The Kruskal–Wallis test and the model's loadings are used to classify the variables.

RESULTS

The different scion/rootstock combinations have contrasting grafting success rates at each key point of the process

Twenty-two different scion/rootstock combinations of grapevine were grafted, and their development was assessed overtime at key points of the process. The vast majority of the grafts successfully developed a visible proliferation of callus cells (Supplementary Figure 1A) at the graft interface during the stratification period. Callus was well-developed for 90 % to 100 % of the grafted vines in all the combinations except for Ugni blanc 482/RSB1 141, in which about 83 % of the grafts had a well-developed callus (Figure 1; Supplementary Figure 1B). After plantation in the nursery, visual assessment of plants in May revealed the appearance of differences in growth between the different combinations. For some combinations, such as Ugni blanc 482/RSB1 141 and Ugni blanc 478/SO4 762, a low percentage of plants had begun to grow and develop (Figure 1). For some scion/rootstock combinations, there was normal development at the beginning of spring, with the development of a healthy shoot, but some plants began to develop abnormally with yellowing leaves in the summer, and finally, many of these plants died in the nursery (Supplementary Figure 2): this was the case for Bourboulenc 541*/5BB and Macabeu 789*/5BB and to a lesser extent for Grenache 363*/5BB, Cinsaut 67*/5BB and Negrette 581/140Ru (Figure 1). By contrast, some combinations maintained good development through the year and had high grafting success rates (such as Merlot 343/SO4 and Pinot noir 828 or Pinot noir 374/110R (Figure 1)).0

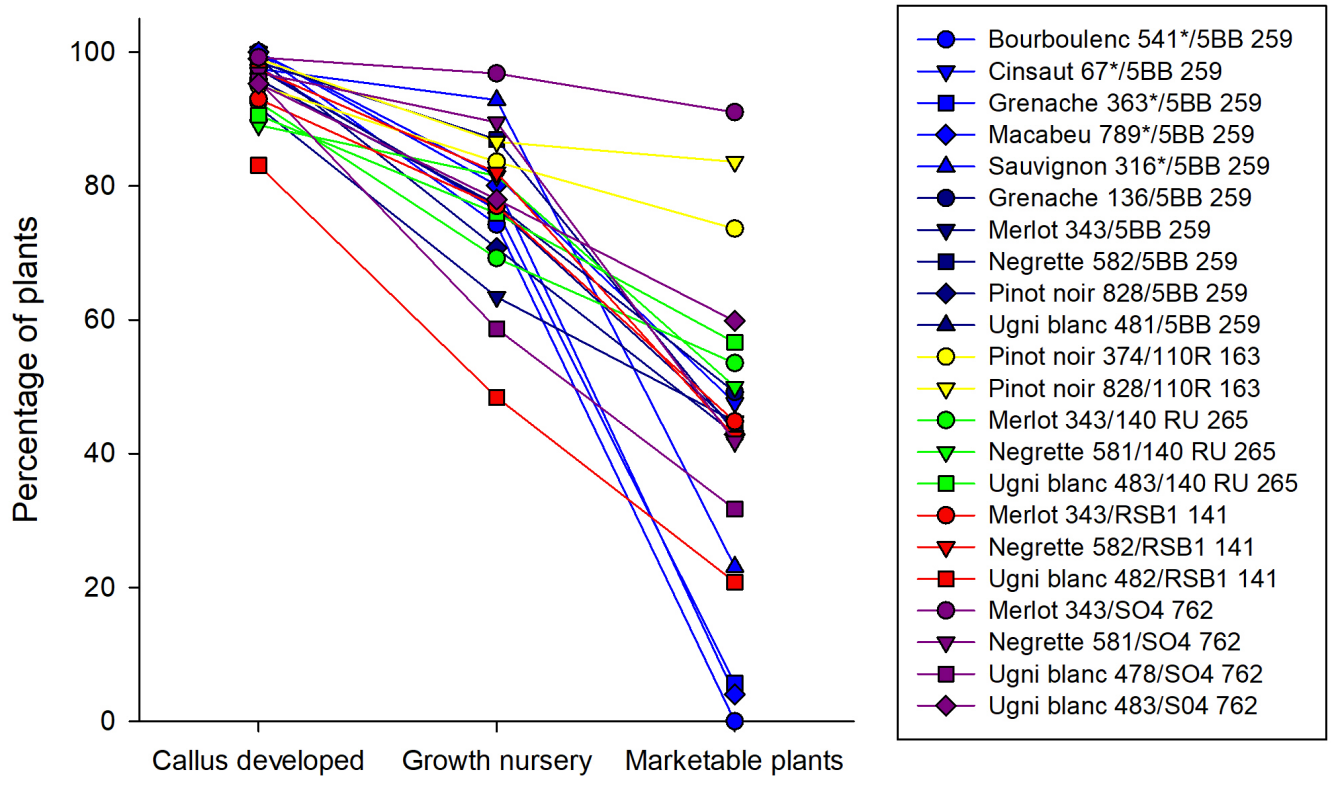


FIGURE 1. The development of 22 scion/rootstock combinations of grapevines during the first year after grafting. The percentages of plants with well-developed callus just after stratification (= Callus developed), that had started growing in the nursery approximately two months after grafting (= Growth nursery), and that were marketable according to the French regulatory criteria at the end of the growing season nine months after grafting were calculated for each combination. The percentages given correspond to overall averages.

** scion clones known to be infected with GLRaV-2.*

We can thus distinguish four types of behaviour:

1. Scion/rootstock combinations that maintained good development through the year and had high grafting success rates that could be qualified as "trouble-free" combinations.

2. Scion/rootstock combinations that showed less good growth in the nursery and poor grafting success at the end of the growing season (such as Ugni blanc 482/RSB1).

3. Scion/rootstock combinations that appeared to grow well at the beginning of the nursery and then died during the growing season (the cases of Grenache 363*, Macabeu* and Bourboulenc* grafted onto 5BB).

4. Scion/rootstock combinations that appeared to grow well in the nursery appeared healthy before being uprooted but gave poor grafting success rates after the thumb test, such as Négrette 581/S04.

For Macabeu 789* and Bourboulenc 541* grafted with 5BB, too few grapevines were still alive after the year in the nursery to be used for further phenotypic characterisation.

For most of the scion/rootstock combinations, there is a relationship between the number of the grapevines that developed early in the nursery and the number of marketable plants produced at the end of the year (Figure 2). Nevertheless, some scion/rootstock combinations are an exception. It is notably true for the combinations when the scion is infected with GLRaV-2 and it is grafted onto 5BB, and for all the combinations with a Négrette scion (independent of the rootstock used). Interestingly we can also observe that 5BB does not give very good results even when grafted with a non-GLRaV-2 infected scion.

Unmarketable grafts generally have poor development.

When all the marketable and unmarketable grafts are compared, globally unmarketable grafts had shorter lengths of lignified stem produced from the scion bud (Figure 3A), smaller scion diameters at the 2nd internode (Figure 3B) and fewer large and small diameter roots (Figure 3C) when compared to marketable grafts. The diameter of the rootstock wood was not significantly different between marketable and unmarketable grafts (Figure 3B). The most frequent causes of a graft being classified as unmarketable are that it failed the "thumb test" or it failed both the "thumb test" and had poor root development (Supplementary Figure 3).

Differences in grafting success are associated with other external plant phenotypes

For two combinations combining GLRaV-2 infected scions grafted onto 5BB (Macabeu 789*/5BB and Bourboulenc 541*/5BB), almost all the vines died before uprooting. No phenotypic characterisation was done because of the very small number of plants produced.

The remaining 20 scion/rootstock combinations showed variation in the values for the criteria used to evaluate the grafted grapevine development (Figure 4). Hierarchical clustering of the phenotypes of the different scion/rootstock combinations separated two particularly poorly developed scion/rootstock combinations (Grenache 363* and Sauvignon 316* grafted with 5BB) and two further clusters of high and low grafting success (Figure 4). Obviously, the different scion/rootstock combinations which produced a high percentage of marketable plants had higher scores in the criteria used to select marketable plants (namely the

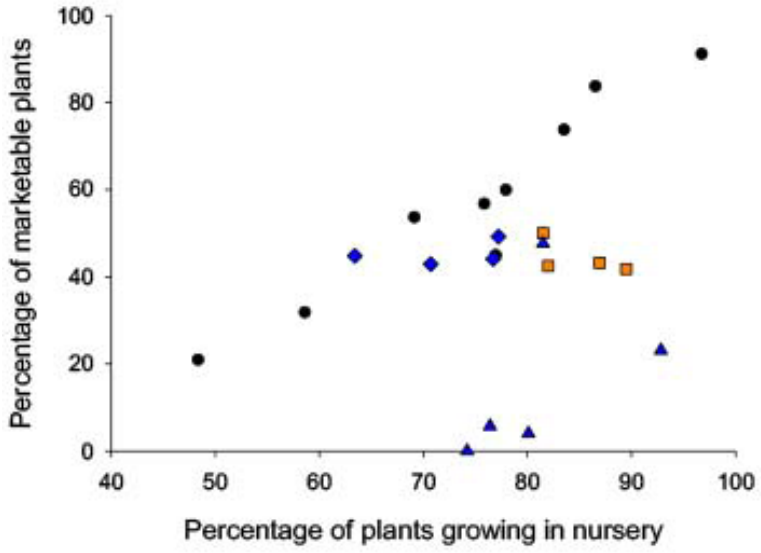


FIGURE 2: Relationship between the development of grafted plants in the nursery (evaluated in May) and the grafting success at the end of the year in 22 scion/rootstock combinations of grapevine. Blue triangles: grafts with scions infected by GLRaV-2 grafted onto 5BB; blue diamonds: grafts 5BB rootstocks and non-infected scions; orange squares: grafts with Négrette as scion; black circles: the remaining scion/rootstock combinations. The percentages given correspond to overall averages.

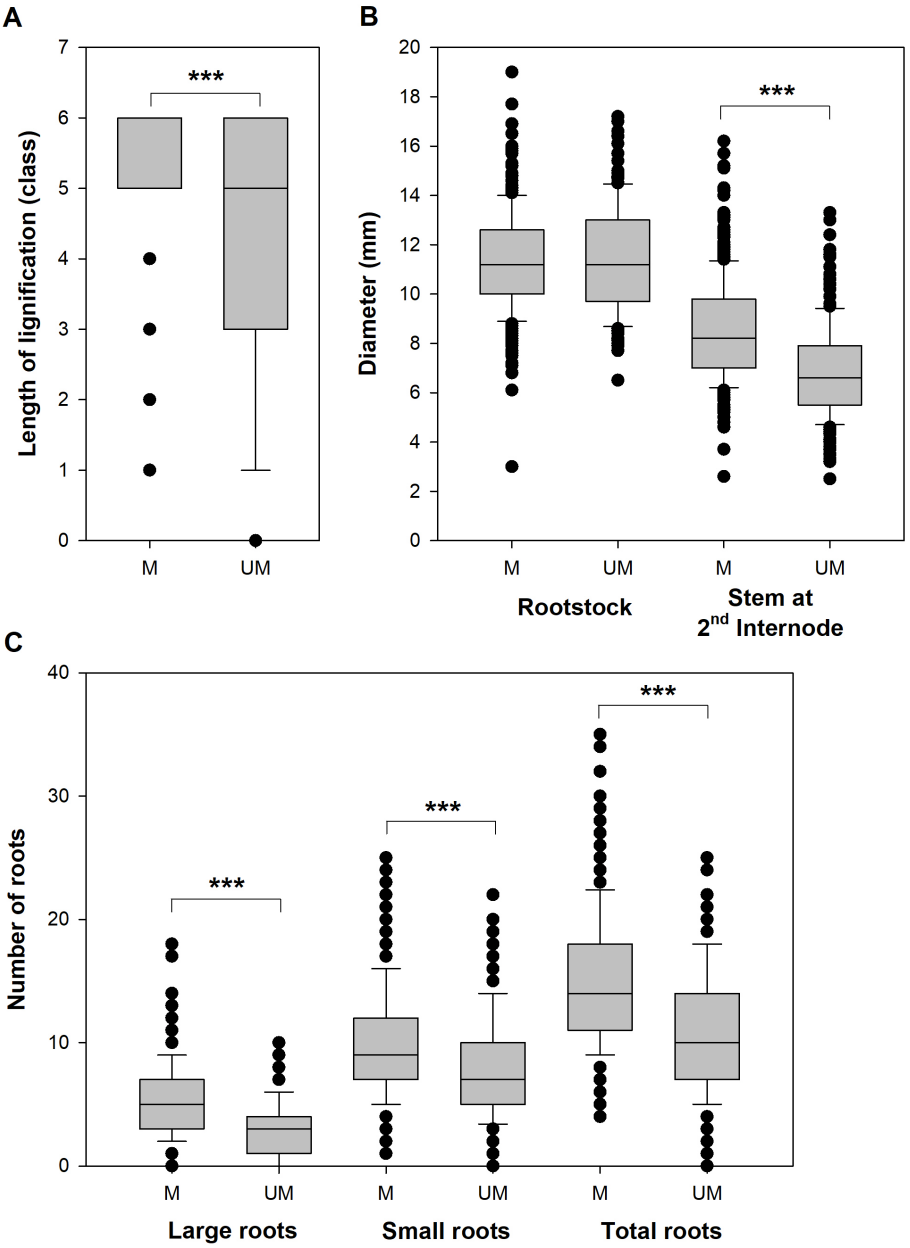


FIGURE 3. Box plots of the difference in (A) lignified stem length, (B) rootstock wood and stem at the 2nd internode diameter and (C) number of roots between 415 marketable (M) and 183 unmarketable (UM) grapevines. The boxes of the box plots represent the first and third quartiles, the thick line represents the median, the whiskers represent the 10th and 90th percentiles, and the circles represent the outliers. Significant differences were tested with a Mann–Whitney Rank Sum Test.

thumb test, the distribution of roots around the trunk and the length of the lignified stem, Figure 4). Other criteria such as root number, stem and rootstock wood diameter were not so clearly associated with the percentage of marketable plants (Figure 4).

The correlation between the different criteria showed that the percentage of marketable plants was most strongly correlated with the thumb test but also positively correlated with the length of lignified stem (Figure 5). Rootstock diameter was negatively correlated with the percentage of plants that pass the thumb test, are marketable, have a long length of lignification and a homogenous distribution of roots (Figure 5). This could be related to the diameter differences between the rootstock genotypes: 140 RU had a small diameter but produced a high proportion of marketable plants; whereas the rootstock 5BB had a large diameter and produced a high proportion of unmarketable plants in this experiment because many of the grafts made with scions infected with GLRaV-2 (Figure 5). The number of small diameter roots was negatively correlated with callus regularity (Figure 5).

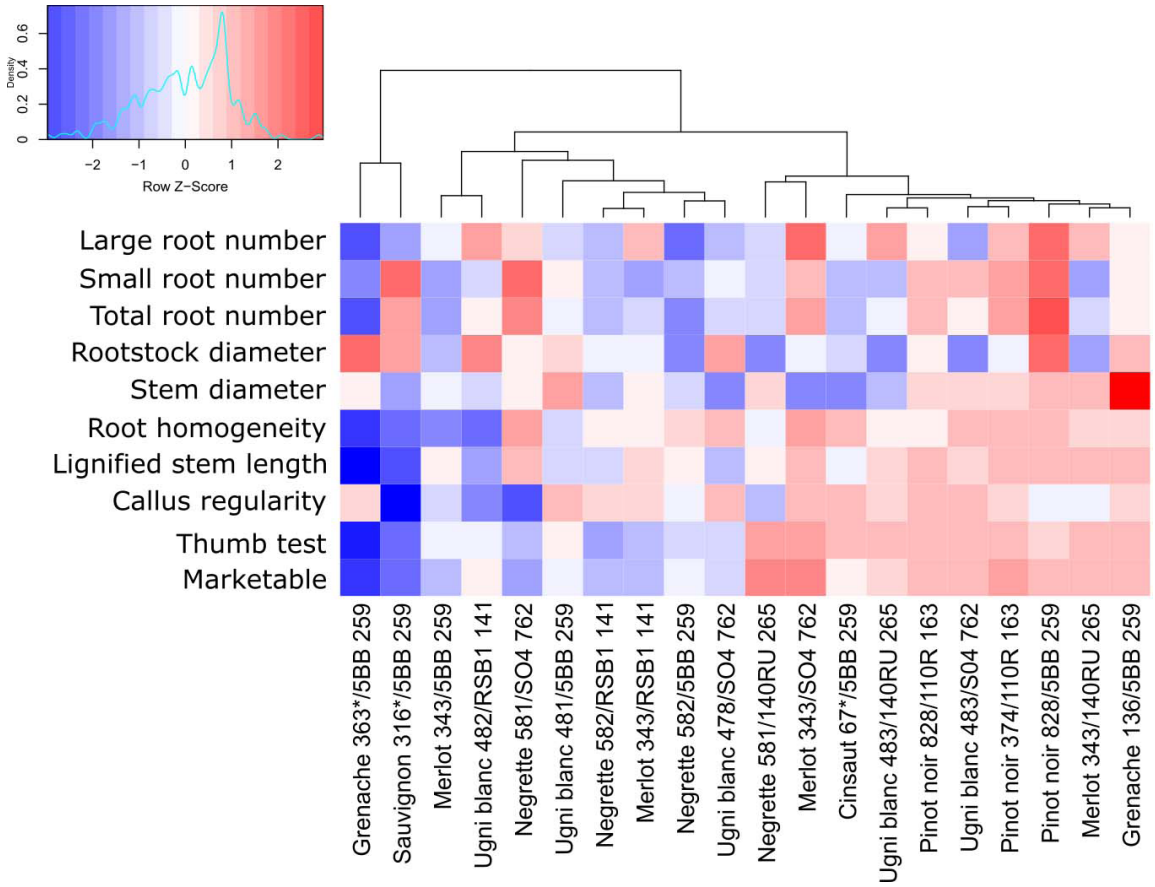


FIGURE 4. Heatmap of the mean of different variables used to quantify grafted plant quality in different scion/rootstock combinations of grapevine. Stars indicate that the scions were infected with GLRaV-2.

Multifactorial analysis of criteria used to select good quality grapevine grafts

A factor analysis of mixed data was used to analyse the data set because the individuals were described by both qualitative (except marketable/unmarketable) and quantitative variables; the first two dimensions explained 56.5 % of the phenotypic variance (Figure 6A). In general, the good quality, marketable grafts were towards the positive side of dimension 1 and to the negative side of dimension 2, whereas the poor quality, unmarketable plants were towards the negative side of dimension 1 and the positive side of dimension 2. The correlation between the quantitative and qualitative variables and the principal dimensions are shown in Figure 6B and 6C, respectively; the variables total number of roots, marketable, the thumb test, total number of large roots, root homogeneity and length of stem lignification contributed most the dimension 1, whereas rootstock diameter, the number of small and total roots, the thumb test, callus regularity and marketable contributed most to dimension 2. A PLS-DA analysis was then used with the constraint marketable and unmarketable to identify which external variables could predict whether a graft is marketable or not (Figure 7). The PLS-DA did not explain a large amount of variance, only 11.1 % (Figure 7A), and the variables with the highest predicted effect were the number of large roots and length of the lignification (Figure 7B).

DISCUSSION

Multiple possible causes of poor grafting success

Most of the commercialised grapevines in France are omega grafted. This method allows mechanisation, large-scale production and often a good success rate. Nevertheless, the success rate can vary depending on the nursery process and scion/rootstock combinations. In this study, grafting success was evaluated in 22 scion/rootstock combinations of grapevine that presented from 0 to 91 % grafting success.

Very poor grafting success potentially caused by a virus was assessed in scion/rootstock combinations associating the rootstock 5BB and different scion varieties known to be infected by GLRaV-2. Grafting failed completely for one combination (Bourboulenc 541*/5BB) and nearly completely for Macabeu* 789/5BB and Grenache 363*/5BB with 4 and 6 % of the grafted grapevines being classed as marketable, respectively. By contrast, two other combinations, which combine GLRaV-2 infected scions (Sauvignon 316* and Cinsault 67*) with the rootstock 5BB,

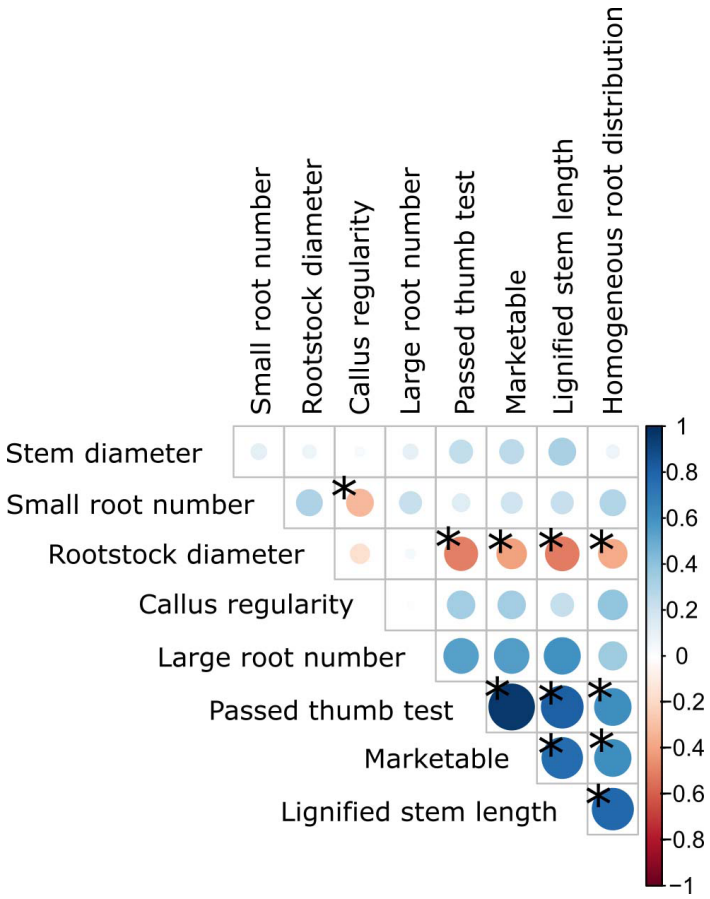


FIGURE 5. Correlation matrix of different variables used to quantify grafted plant quality in different scion/rootstock combinations of grapevine. Stars indicate statistically significant correlations, $p < 0.05$.

produced reasonable percentages of successfully grafted plants, 23 and 45 %, respectively.

For all these five combinations including GLRaV-2 infected scions, the percentage of grafts with a well-developed callus was as high as in the non-infected ones (between 97 and 100 %): this step did not appear to be affected by the virus presence. A more precise comparison can be made between two combinations involving the same variety with clones infected or free of GLRaV-2: Grenache 363*/5BB and Grenache 136/5BB. These two scion/rootstock combinations had similar levels of callus development (98 % and 96 % of the grafts produced well-developed callus, respectively) and early growth in the nursery (76 and 77 % of the grafts were growing well in the nursery approximately two months after grafting) suggesting that early growth was not affected by this virus. By contrast, important differences between these two combinations were observed in the grafting success at the end of the year: Grenache 136/5BB, which is free of GLRaV-2, produced 49 % of marketable grafts, whereas Grenache 363*/5BB, infected with GLRaV-2, produced only 6 % of marketable plants. All the grafts considered unmarketable failed the thumb test (data not shown). These results indicate that GLRaV-2 infection might reduce vascular connections in grafts with Kober 5BB. This is consistent with what was previously described by Greif *et al.* (1995), who first described this incompatibility: a rapid decline was observed 3 to 6 months after the green grafting of GLRaV-2 infected varieties onto Kober 5BB. Moreover, grafting with scions infected with this virus has been shown to cause a hypersensitive response in certain rootstocks, including 5BB, which results in the death of vascular elements and plant death (Rowhani *et al.*, 2017). The impact of viruses on vascular connection was also observed by anatomical studies realised onto micro-grafted vines. The vascular connection could not be established when scions of Cabernet Franc (co-infected with GLRaV-1 and Grapevine Virus A) were grafted onto four rootstocks compared with healthy control (Cui *et al.*, 2019).

Surprisingly, there was a great variation between the grafting success of the five combinations involving GLRaV-2 infected scions (0 to 48 %). One of the hypotheses to explain why in some cases, a reasonable percentage of marketable plants was produced could be that not all the buds in the scions were infected by the virus as grapevine viruses are known to be heterogeneously distributed in the canes (Kominek *et al.*, 2009). Furthermore, the strains of GLRaV-2 in these scion varieties might also be different and it was shown that some strains (RG and PN) were more likely to induce the hypersensitive response in 5BB (as reviewed by Rowhani *et al*., 2017).

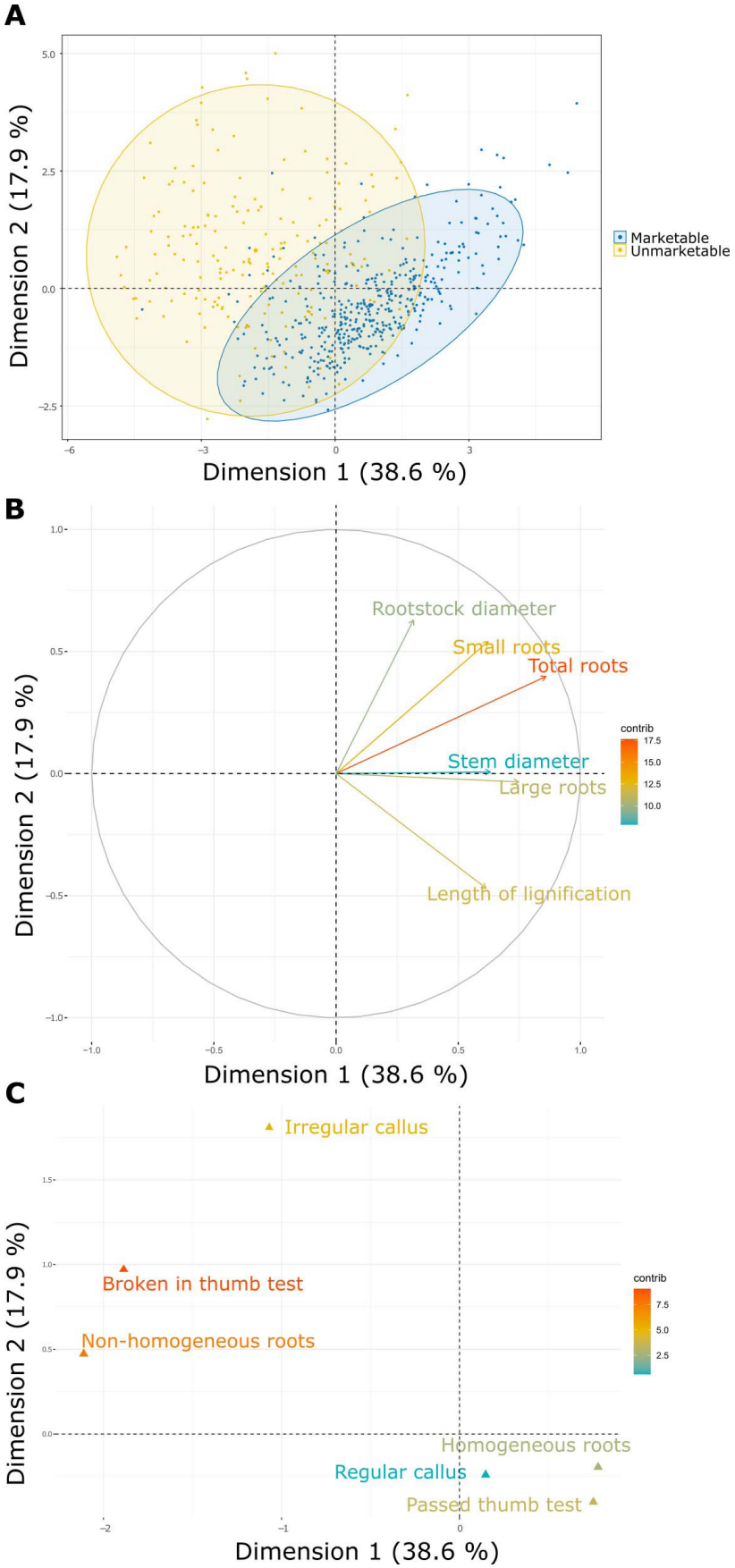


FIGURE 6. Factor analysis of mixed data (FAMD) was used to describe different scion/rootstock combinations of grapevine; (A) plot of the individuals, marketable and unmarketable grafts shown in blue and yellow respectively, this variable was not used in the FAMD, and (B) quantitative and (C) qualitative variable categories used for the FAMD.

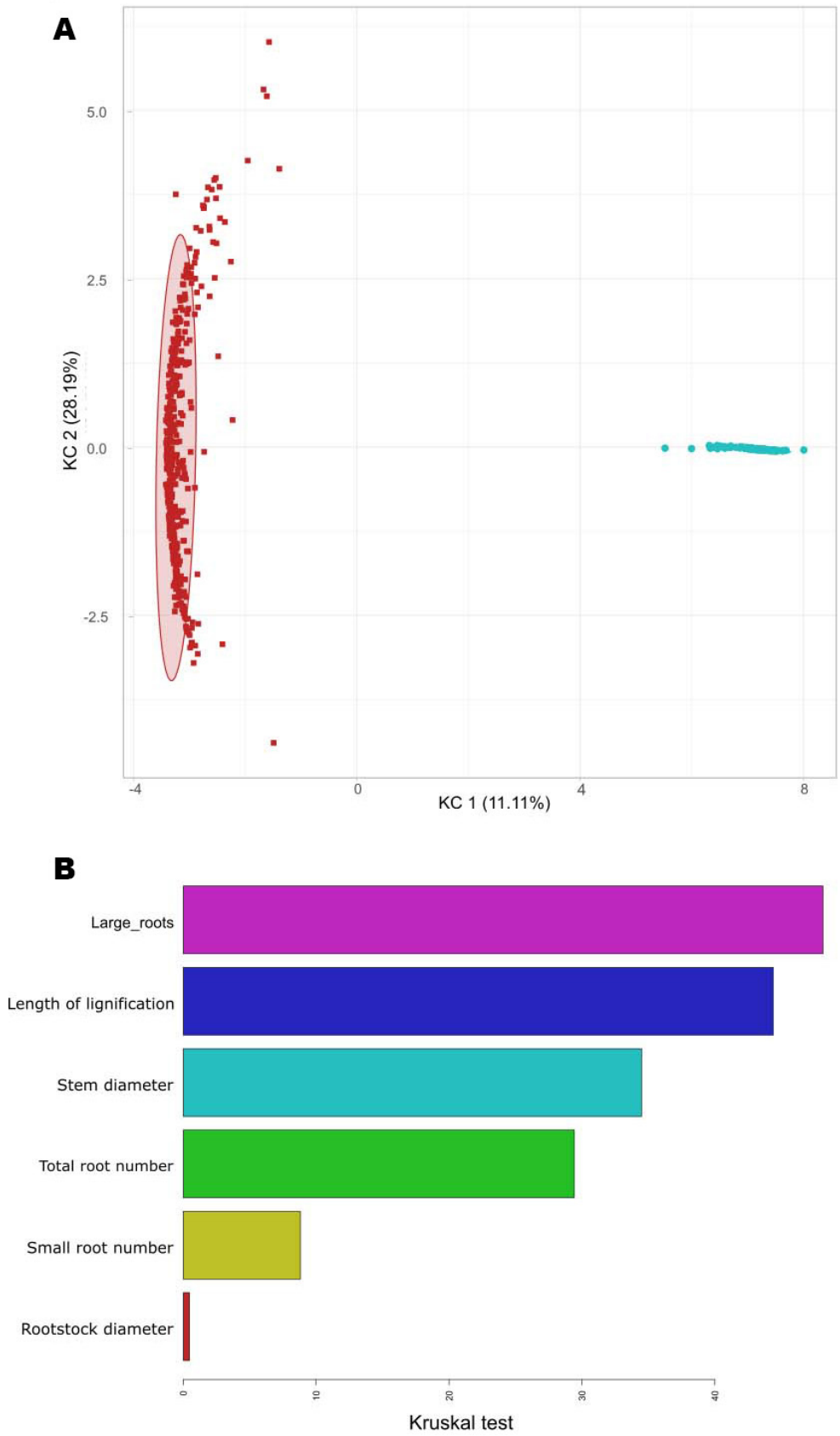


FIGURE 7. A. PLS-DA analysis of external variables with the constraint unmarketable (red) and marketable (turquoise) grafts, B. the ranking of the predictive variables used in the analysis.

In addition to these well-known and previously characterised virus-based incompatibilities, some scion/rootstock combinations not defined as incompatible are nevertheless recognised by nursery workers as "difficult to graft". It is notably the case of Ugni Blanc/RSB1 and Négrette/140 RU (P. Bloy and O. Yobregat, IFV, personal communication). For the former combination Ugni Blanc/RSB1, generally, the proportion of plants growing in the nursery and marketable plants at the end of the year was low in our study, although the callus development at early stages was good. The problem for this combination seems to be related to early events in the grafting process as the percentage of healthy plants is low at only two months after grafting, which is different to the incompatible viral combinations described above. The combination Négrette/140 Ru was selected because nursery workers describe it as problematic. In this case, the problems do not appear during the nursery process but later often during the first year after plantation (O. Yobregat, IFV, personal communication). Contrary to Ugni Blanc/RSB1, the development of Négrette/140 Ru seemed normal in the early stages in the nursery. However, in all the grafts we studied with a Négrette scion, a significant proportion of the grafts developed well in the nursery but did not pass the thumb test. This could suggest that Négrette is the cause of some developmental or graft union failure problems. In both these problematic combinations, the causes of poor grafting success appear to be different because the problems arise at different developmental stages. Concerning all the other combinations, we can observe that we have a direct relationship between the percentage of grapevines that develop early in the nursery and the plants that pass the test. This indicates that the early steps of the callus formation are certainly for the "normal" scion/rootstock combinations, the key step to determine overall grafting success.

Evaluating grafted plant quality

The thumb test remains the major criteria for discriminating marketable and unmarketable grafted grapevines today; most of the grafted grapevines are eliminated following this test (P. Bloy, IFV, personal communication): it is also what was observed in this study. It is quite rare that grapevines pass the thumb test and do not pass the test for roots and lignified stem length, confirmed by our results (Supplementary Figure 3). In our experiment, grafted grapevines were eliminated mainly due to failing the thumb test criterion (84 %) with or without failing other criteria (47 and 37 %, respectively). Only 6 % of the plants passed the "thumb test" and were eliminated on root development criteria. No plant was eliminated only on the shoot criteria. We observed that 10 % of the grapevines were eliminated onto other criteria such as wounds on the rootstock, etc.

The first limit of the thumb test is that it is a non-quantifiable process because of the lack of mechanisation and its reproducibility is low. As it is carried out manually, the force exerted can be highly variable depending on each person and can vary throughout the day. In this study, this test was performed by the same experienced person to limit the variability as much as possible.

Given the central role of this criterion in the evaluation of grafts, the possibility to replace it with an objective and potentially automatable test is of interest. Few studies have been carried out to assess the possibility of mechanising the thumb test or replacing it with something totally different (Pisciotta *et al.*, 2017; Tedesco *et al.*, 2020). Given the huge number of plants that need to be screened by commercial nurseries, optical screening would seem to be the most appropriate high-throughput method. Although the diameter of the scion and rootstock next to the graft interface has been frequently cited as a criterion of graft compatibility (e.g., Gargin and Altindisli, 2014 and references therein), Tedesco *et al.* (2020) found that it resulted in contradictory conclusions.

In this study, we have evaluated the ability to use other visual criteria to replace this thumb test. Globally, unmarketable plants have poorer development than marketable plants with shorter lignified stems, thinner stems and fewer roots. In this study, the length of the lignified stem and the number of large roots are the main relevant indicators to discriminate between marketable and unmarketable grafted vines.

The correlations between these criteria are low, indicating that a single one of these criteria cannot be used to select good quality plants, and none of these criteria is strongly correlated with the "thumb test" and therefore cannot replace it. Thus, the ability to use new criteria to separate marketable and unmarketable grafts is low in the PLS-DA; it seems that evaluating grafting plant quality by external visual criteria will be challenging and requires further work.

And finally, what is a good quality grafted grapevine?

Even though the thumb test is the most discriminating criterion to eliminate not marketable grafted grapevines, whether this criterion has any importance in terms of the success of vineyard plantations or long-term plant survival has never been scientifically evaluated. It seems logical to assume that the mechanical resistance is linked to the quantity of wood developed, its regular distribution over the graft union and the quality of xylem lignification. Nevertheless, this test may be insufficient, allowing plants with poor quality junctions to pass. Conversely, it could be too stringent, eliminating plants that would be able to develop harmoniously. Finally, the impact of doing the thumb test on grafted plants is not known; it might lead to micro-fractures in the tissues that could be harmful in the long term.

New approaches allowing us to characterise the organisation of the graft interface of the marketable or unmarketable grafts would be of great interest. It is notably the case of new imaging tools such as magnetic resonance imaging and x-ray tomography. The use of these techniques has already been tested in grapevine and has shown promising results (Bahar *et al.*, 2010; Milien *et al.*, 2012).

Despite the effort made to assess grafted plant quality before planting, to our knowledge, there have been no peer-reviewed scientific studies on the long-term impact of planting what may be considered as poor-quality grafted plants in a vineyard. However, it seems reasonable to assume that

planting grafts with fragile, easily broken graft interfaces, poorly developed roots, and few reserves will not be good for the future development of the plant. Unfortunately, the low cost of the planting material in viticulture has led to the underestimation of the value of good quality plants, which is a real impediment for the investment and innovation in the nursery industry today. Given the importance of the current interest of grapevine decline, better knowledge of what is a "good-quality grafted vine" becomes an urgent need.

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