Techniques to study graft union formation in grapevine

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Abstract:

Context and purpose of the study – Grapevines are grown grafted in most viticultural regions. Grapevine rootstocks are either hybrids or pure species of different American *Vitis spp.* (particularly *V. berlandieri*, *V. rupestris* and *V. riparia*), which were primarily used to provide root resistance to the insect pest Phylloxera. In addition to Phylloxera resistance, grapevine rootstocks were also selected in relation their resistance to various abiotic stress conditions. Future rootstocks should have the potential to adapt viticulture to climate change without changing the characteristics of the harvested product. However, high grafting success rates are an essential prerequisite to be able to use them with all the varieties. The objective of this work is to develop quantitative techniques to characterize graft union formation in grapevine.

Material and methods – The development of grafts of different scion/rootstocks of grapevine was studied during the first few months after grafting. The quantity of callus produced (fresh and dry mass) and the mechanical strength of the graft union were quantified in five scion/rootstock combinations 5-6 weeks after grafting. The scion/rootstock combinations studied were homografts of the typical grapevine scion genotype, *Vitis vinifera* cv. Pinot Noir (PN), and homografts of two rootstocks, *V. riparia* cv. Gloire de Montpellier (RGM) and the *V. berlandieri x V. rupestris* cv. 140 Ruggeri, and two hetero-grafts, PN/RGM and PN/140Ru (n=27). We also used x-ray tomography to study functional xylem vessels by labelling functional vessels with the contrast agent Iohexol. This protocol was optimised in three omega grafts of *V. vinifera* cv. Tempranillo grafted onto the rootstock *V. berlandieri x V. rupestris* cv. 110 Richter. Grafts with solid and resistant graft unions were selected after one year of growth in a nursery and grown in a greenhouse until approximately 10 leaves had appeared to drive the movement of iohexol in the xylem. Scans were analyzed with the computer programs Fiji/ImageJ and Imaris.

Results - Equipment to quantify the mechanical strength of the graft union was developed and tested on different scion/rootstock combinations to determine the suitability of this technique to quantify graft union development. The quantity of callus produced at the graft interface is different between the tested genotypes and was not necessarily related to the mechanical strength of the graft union. Three-dimensional reconstruction of x-ray tomography images allowed us to visualize the vessel connections between the scion and rootstock, and this knowledge will be used to develop protocols to quantify xylem vessel connections using high-throughput methods.

Significance of the study – Difficulties in quantitatively phenotyping the different steps of the graft union formation have considerably delayed the identification of the genetic determinants of grafting success in all the plant species. In this study, we are developing various quantitative methods to overcome this bottleneck with the objective to be able to characterize the genetic mechanisms involved in graft union development in grapevine.

Keywords: Grapevine, xylem vessels, grafting, callus, mechanical strength, Tomography-RX.

1. Introduction

Graft compatibility is the ability of the assembled scion/rootstock to form and sustain a functional graft union. However, in many cases, the two genotypes do not always form a successful graft and the graft interface is associated with problems of necrosis, insufficient vascular connections between the scion and rootstock, and poor plant survival, which is often termed graft incompatibility. Even though graft incompatibility is currently rare in viticulture, this may not be the case in the future with of the development of new genotypes adapted to future climatic scenarios. Having methods to evaluate graft union formation in new scion/rootstock combinations will facilitate the selection of new grapevine genotypes.

Graft union formation is a complex process that includes an initial wound response followed by the exudation of pectin-like compounds to adhere the scion and rootstock together. Then, the proliferation of parenchymal cells forms a callus that will serve as a bridge between the two partners. Finally, the vascular tissues connect between the scion and the rootstock (Loupit *et al*., 2023). Today, there is no simple method to characterize quantitatively the graft union of grapevine. In general, the manual 'thumb test' (pressure is applied to the graft interface to make sure that it does not break easily) is used at the end of the nursery process to evaluate the quality of the graft union. This test appears to be difficult to replace with alternative measurements (Carrere *et al.*, 2022). In herbaceous grafts, break tests have been used to assess the mechanical strength of the graft union in tomato and pepper grafts (Thomas *et al*., 2021). Other studies have quantified the mechanical strength of the graft interface for example in *Arabidopsis thaliana* hypocotyl grafts (Melnyk *et al*., 2018) and *in vitro* stem grafts of tobacco (Kawakatsu *et al.*, 2020), but these tests have not been used to quantify differences between different scion/rootstock combinations. The processes that contribute to the mechanical resistance of the graft union are not known. Some hypotheses include the exudation of extracellular pectins, the formation of callus and the connection of the vascular tissues, particularly the xylem, which is lignified and provides much of the tensile strength of plant stems (Liu *et al.*, 2018).

The objective of this work was to develop techniques to assess the different processes involved in graft union formation in grapevine. Firstly, we quantified the mechanical strength of the graft union by measuring the force required to break the attachment between the scion and rootstock. Secondly, we wanted to determine whether the mechanical strength of the graft union is related to the quantified the quantity of callus produced at the graft interface. Thirdly, we used a xylem loaded contrast agent to examine functional xylem connections between the scion and rootstock with x-ray computed tomography (µCT).

2. Material and methods

2.1 Quantifying the mechanical resistance of graft union and callus formation at the graft interface two months after grafting

Homografts of the typical grapevine scion genotype, *Vitis vinifera* cv. Pinot Noir (PN), and two rootstocks, *V. riparia* cv. Gloire de Montpellier (RGM) and the *V. berlandieri x V. rupestris* cv. 140 Ruggeri, were made as well as the hetero-grafts PN/RGM and PN/140Ru. The grafts were made with an omega table-top grafting machine, stratified in a crate containing 1 cm of water place in a room with high humidity at 28 °C (at the INRAE grapevine grafting facility in the EGFV). After stratification, the grafts were transferred to a greenhouse for one month. To quantify the mechanical force required to break the graft union, we assembled a crank bench stand with two clamps to attach to the each end (scion and rootstock) of each graft. We continuously measured the mechanical resistance of the graft interface to the breaking point with a SAUTER FS 2-500 force gauge (Sauter GmbH, Germany). After breaking the graft interface, we removed callus tissue with a scalpel from the graft interface, and dried it in an oven at 80 °C until it reached a constant weight that was measured. Statistical analysis and graphs were done using SigmaPlot 11.

2.2 µCT analysis of xylem connections between the scion and rootstock one year after grafting

Vitis vinifera cv. Tempranillo grafted onto the rootstock *V. berlandieri* x *V. rupestris* cv. 110 Richter. These plants were grafted with an omega grafting machine and grown in a nursery in Navarra, Spain, for one growing season and uprooted in the winter. Three plants considered good quality (i.e. had mechanically resistant graft unions) were stored in a cold room and then the grafts were planted in a greenhouse irrigated daily. After two months, when the plants had approximately 10 leaves each they were used for X-ray tomography analysis.

We used the EasyTom 150 (RX solutions) µCT facilities of the Institut des Sciences de l'Évolution de Montpellier, France. We used imaging with an average spatial resolution of 29 µm/voxel. We first made a control acquisition without iohexol for each plant. Then, the roots were underwater and immersed in an iohexol solution at 60 mg/mL for 24 h. The grafts were place in a growth chamber so that the transpiration stream could label functional xylem vessels that cross the graft interface. We made a second acquisition after 24 h of iohexol loading with the same µCT parameters. Therefore, for each plant, two acquisitions, at t0h and at t24h, were done.

The aim was to reconstruct in 3D the functional xylem network. The 3D images collected at t0h and t24h on the same plant were aligned using the FIJIYAMA plugin (Fernandez & Moisy, 2020) in Fiji/ImageJ (Schindelin *et al.*, 2012), using default parameters. Subtracting the t0h image from the t24h allowed us to identify specifically which vessels contained iohexol and were therefore functionally transporting xylem sap form the root to the shoot. Then iohexol-labelled xylem vessels were segmented using Weka segmentation (a Fiji plugin producing binary pixel classification). The channel corresponding to iohexol-filled vessels can then be extracted from the file to a binary stack. Finally, to visualize the organization of the iohexol-labelled vessels at the graft interface, the IMARIS software was used or 3D viewer in Fiji.

3. Results and discussion

3.1. *The mechanical resistance of the graft interface is not related to callus proliferation*

Two months after grafting grapevine, a force of approximately 200 N is required to break the graft interface (Figure 1A). There was little variation in the force required to break the graft interface between the five different scion/rootstock combinations studied (Figure 1A). This is the first time that the mechanical resistance of the graft interface has been compared between different scion/rootstock combinations; further work will be done to quantify the mechanical resistance of the graft interface at other stages of development to determine whether this measurement is suitable to evaluate genetic variation in graft union formation.

The quantity of callus produced at the graft interface varied significantly between the different scion/rootstock combinations with combinations containing the 140Ru genotype having high callus biomass at the graft interface than the other genotypes (Figure 1B). The rootstock 140Ru is known to produce large bulging graft union in the field (http://plantgrape.plantnet-project.org/), which could be related to this early production of callus.

The mechanical resistance of the graft interface (Figure 1A) does not appear to be linked to the quantity of callus produced at the graft interface in two-month-old grafts (Figure 1B) suggesting that other tissues contribute to the mechanical resistance of the graft union in this early stage.

3.2. *µCT analysis reveals the complexity of the xylem connections between the scion and rootstock in grafted grapevine*

X-ray computed tomography allows us to visualize inside plant tissues by providing information on tissue architecture in relation to X-ray attenuation by different tissue types. The grey levels in a µCT image correspond to the proportion of x-rays scattered or absorbed as they pass through the sample. We can identify different stem tissues in µCT images of grapevine, such as, the pith, empty xylem vessels, xylem parenchyma, phloem and bark (Figure 2A, C). This is in agreement with previous studies on the use of µCT in grapevine (Brodersen, 2013; Brodersen *et al.*, 2013; Milien *et al.*, 2012; Pratt & Jacobsen, 2018).

By comparing the images taken at t0h and t24h (before and after loading a contrast agent to the roots), we can see that iohexol has labelled function xylem vessels at the graft interface of grapevine (Figure 1B and D), suggesting that iohexol can label functional xylem vessels in grapevine grafts. Iohexol has been successful used to label functional xylem vessels in many species including grapevine (Pratt & Jacobsen, 2018), but has not previously been used to study xylem connections between the scion and rootstock in any plant species. The optimization of Image processing techniques and xylem vessel segmentation (Figure 1E) allowed the visualization of the complex network of xylem connections that form between the scion and rootstock one year after grafting (Figure 1F). Our next objective is to quantify these xylem vessels so that we can compare xylem vessel formation in different scion/rootstock combinations.

4. Conclusions

Graft union development is difficult to phenotype quantitatively in all plant species (Loupit *et al.*, 2023). The objective of this work was to develop phenotyping techniques to assess quantitatively different aspects of graft union formation in grapevine, namely, the formation of a solid, mechanically resistant graft union and the quantification of the callus production. We found that the mechanical resistance of the graft union of grapevine two months after grafting is variable. No significant genotype-specific differences were observed in this study, but further scion/rootstock combinations should be tested and at different developmental stages. Callus formation at the graft interface is easy to measure and depended on the genotype studied, but it is not clear how callus cell formation is related to grafting success in grapevine. The combination of µCT and a xylem-loaded contrast agent is able to xylem vessels connecting the scion and rootstock in grafted grapevines, but quantifying xylem vessel connectivity is more challenging and should be a priority for the future.

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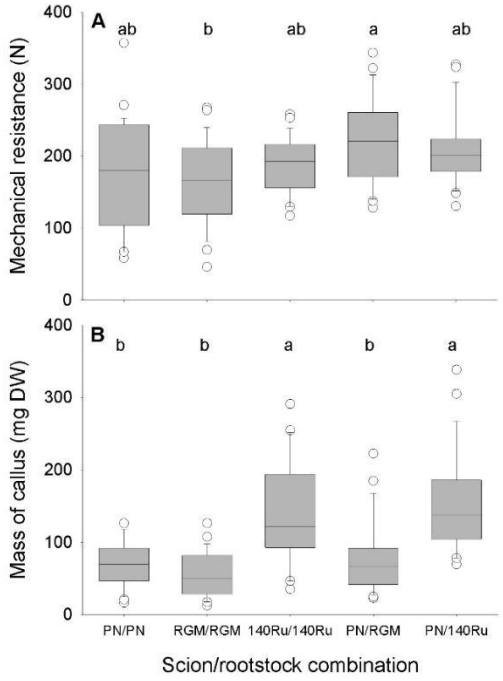

Figure 1. Box plots of **(A)** mechanical resistance of, and **(B)** callus formation at the graft interface of grapevine in five scion/rootstock combinations. Significance tested with ANOVA on ranks, letters represent the results of multiple comparison using Dunn's Method (n = 27). Key to genotypes: PN, *Vitis vinifera* cv. Pinot Noir; RGM, *V. riparia* cv. Gloire de Montpellier; 140 Ru, *V. berlandieri x V. rupestris* cv. 140 Ruggieri.

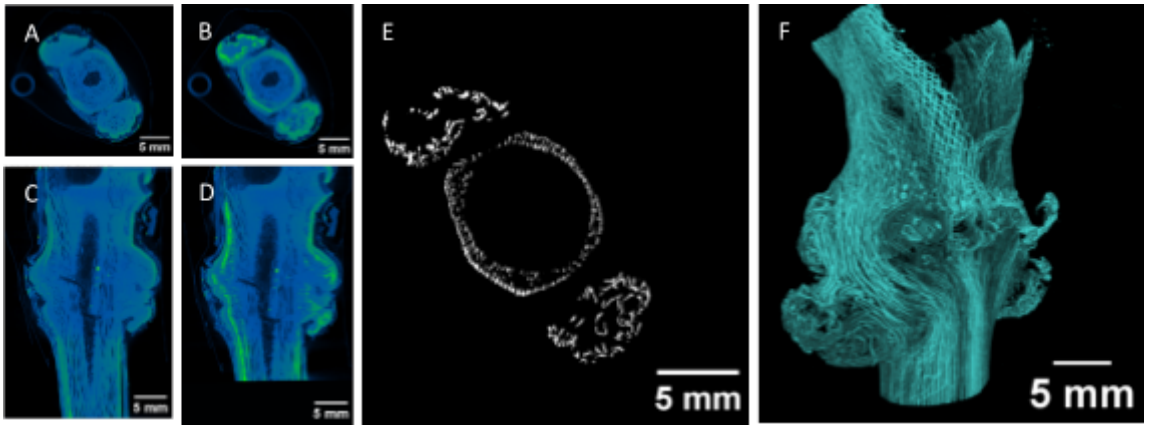

Figure 2. X-ray computed tomography images of the graft interface of grapevine before and after labelling functional xylem vessels with iohexol. False colour images of **(A, C)** before and **(B, D)** after feeding iohexol to the roots of one year old grapevine grafts. The iohexol labelled xylem vessels were isolated and segmented by comparing t0h and t24h **(E)** and then the network of functional xylem vessels was visualized **(F)**.