Evaluation of growing and photosynthetic parameters in response to simple and mixed infections with GRSPaV in plants of *Vitis vinifera* L., "Cabernet Sauvignon" cultivar and study of new strategies of GRSPaV elimination.

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2018
Pontificia Universidad Católica de Chile
Facultad de Agronomía e Ingeniería Forestal

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Thesis
to obtain the degree of

Doctor
en Ciencias de la Agricultura

Santiago, Chile, March 2019
Thesis presented as part of the requirements for the degree of Doctor in Ciencias de la Agricultura, approved by the

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Dedication

To my family,
Juan Pablo

and to the friends who have accompanied me these years.
This work was supported by CONICYT fellowship (21141168/2014) and Laboratorio de Berries of Pontificia Universidad Católica de Chile. We recognize the collaboration of Laboratorio de Bacteriología y Fitovirología of Universidad de Chile and Laboratorio de polímeros of Pontificia Universidad Católica de Chile. Finally, we also thank I+D Vinos de Chile (Project 12CTI-16788-01).
Acknowledgements

I thank everyone who accompanied me on this path. At a professional level I thank the thesis committee and their respective laboratories who have been essential in the development of this work. On a personal level, I thank my family, especially my parents, who have always been supporting and encouraging me, I love them.

Additionally, I would like to acknowledge the friends with whom I have shared during these 5 years. Finally, I thank to Juan Pablo for being with me every day in each of the processes we have lived.
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Chapter 1

General Introduction

*Vitis vinifera* spp. is host of several pathogens such as nematodes, fungi, bacteria and phytoplasmas. Nearly 70 different viruses capable to infect grapevine have been described, giving rise to at least 25 different viral diseases, becoming one of the most susceptible species to virus infections (Martelli, 2014a). At a general level, the development of symptoms for these diseases negatively affect plant propagation, as well as vigor, wood quality and some parameters of agronomic and oenological interest (Mannini et al., 2012; Vișoiu et al., 2000). Natural transmission of viruses occurs through pollen, seeds or insects. However, contaminated plant material is one of the transmission pathways with greatest impact on viticulture, because it allows pathogen spread over long distances and extended periods of time. Therefore, the presence of these microorganism has become widespread in vine crops all over the world, an industry that is characterized by using clonal plant material for grapevine multiplication.

A study performed sampling at the six most relevant regions for the viticulture industry in Chile, revealed the presence of the main causal agents of the following diseases: - Leafroll (GLRaV-1, -2 and -3), Infectious Degeneration (GFLV) and - Rugose wood complex (GVA and GVB) (Fiore et al., 2008). These three viral diseases have the greatest impact worldwide. Also, the most frequent virus detected
in this study was Grapevine Fleck Virus, which was present in almost half of the analyzed samples.

Research by Fiore et al. (2008) also reported the presence of Grapevine rupestris stem pitting associated virus (GRPSaV) for the first time in Chile, which is one of the causal agents of rugose wood complex disease. A subsequent study analyzed phytosanitary status of plants belonging to the most important grapevine cultivars in Chile and detected the presence of GRSPaV in 91% of analyzed samples (personal communication). On an international level, its distribution is extensive and has been reported in a large number of countries throughout the world (Meng et al., 2017).

**Effect of GRSPaV infection on grapevine**

GRSPaV was simultaneously discovered in 1998 by two laboratories after numerous attempts to detect the causative agent of rupestris stem pitting syndrome (Meng et al., 1998; Zhang et al., 1998), belonging to the rugose wood complex disease. This disease was described in injerted grapevines and it is characterized by development of pittings and grooves in the stem of graft and/or rootstock. In more severe cases, it is possible to see the swelling of wood around the area where the union occurred, a symptom closely associated with graft-rootstock incompatibility (Maliogka et al., 2015). The severity of symptoms varies depending on a number of factors such as graft-rootstock combination, variant of virus or environmental factors existing at the time of infection (Maliogka et al., 2015), for example less symptoms are observed in cold or humid climates (Martelli, 2014b).
GRSPaV has been also defined as the causal agent of vein necrosis, a disorder observed in the 110 Ritcher rootstock (Bouyahia et al., 2005). It is a widely distributed disease and has a high level of incidence due to its ability to transmit by asymptomatic plant material. When contaminated plant are grafted onto the 110 Ritcher cultivar, vein necrosis is induced on the underside of the leaf at the base of shoot, to continue to upper leaves and even to the adaxial side of the leaf. More severe stages of this disease induce necrosis in tendrils and shoots, along with growth reduction and even plant death (Martelli, 2014a). However, the identification of GRSPaV as a direct causal agent of this disease has not been confirmed and additional evidence is required.

The vast presence of this virus in asymptomatic plants makes it difficult to establish only GRSPaV presence as the trigger of certain grapevine disorders. Furthermore, there is evidence that the presence of the virus in asymptomatic plants could be innocuous for the plant growth, yield and berry qualities (Reynolds et al., 1997). For this reason, it is increasingly necessary to study the virus effects on grapevines in order to understand its real impact on plant development and viticulture productivity.

In this context, some studies have compared growth and productive parameters of virus-free plants and grapevines infected with GRSPaV. In 1997 Reynolds et al. evaluated parameters such as plant growth, yield and budburst in virus-free and GRSPaV-infected plants in five different cultivars (Kerner, Michurinetz, Okanagan Riesling, Madeleine Sylvaner and Ortega). The evaluations were performed in two locations with different climate conditions, Summerland (arid continental climate) and Sydney (cool maritime climate), during six seasons. Although at local level some
differences were observed, in general, the analyzes indicated that there was no negative effect of GRSPaV infection on the evaluated parameters.

A similar study was carried out with cultivars Albano and Trebbiano Romagnolo grown in northern Italy and inoculated with mixed infections of GRSPaV and other grapevine viruseses. This study showed that, in most plants with multiple viral infections that included GRSPaV, no differences in growth parameters, yield or fruit quality were observed. In contrast, plants with mixed infections that did not included GRSPaV, a reduction in the previously mentioned parameters was found (Credi & Babini, 1997).

More recent studies evaluated similar productive parameters in Savagnin rose cultivar during six seasons in three phytosanitary status: virus-free plants, grapevines with simple infections of GRSPaV and plants with multiple infections of GLRaV-1, GVA and GRSPaV. Results were congruent with other studies, since the plants with simple GRSPaV infections did not show differences on vigor, nor yield in comparison with virus-free plants. However, grapevines samples with three viruses showed a growth reduction close to 20% and a decrease of approximately 50% of the yield (Komar et al., 2010).

In plants of cultivar Bosco infected with GRSPaV the virus did not reduce vigor, yield or plant fertility, nor aspects of the bunch or chemical components of the berry (Gambino et al., 2012). However, it should be noted that in this study, a reduction in berry size of GRSPaV-infected plants was observed. This is an interesting result,
considering that in the same publication it was observed that the highest concentration of GRSPaV was found in this tissue.

Gambino et al. (2012) also analyzed some parameters associated with photosynthetic processes of the plant. A reduction in chlorophyll content in GRSPaV-infected leaves relative to healthy plants was observed. Additionally, an increase in internal concentration of CO$_2$ in the leaf (C$_i$) and in evapotranspiration (E) of infected plants was found at time berry size was approximately 7 mm (EL-31; Coombe, 1995), and a reduction of net photosynthetic rate (P$_n$) when the berry begin to colour and enlarge (EL-35; Coombe, 1995).

A comparison of RNAm levels present in healthy and GRSPaV-infected plants was carried out by Gambino et al. (2012). When comparing the number of differently expressed transcripts by gene category, the group of estress response genes showed a significantly different transcriptional profile. Upon further analyses, it was observed that most of repressed genes were related to biotic stress response, while a large number of genes over-expressed in GRSPaV-infected plants belonged to the abiotic stress response pathway. These uncommon transcriptomic changes induced by GRSPaV, associated with nearly a null effect on photosynthetic and productive plants parameters, led the authors to hypothesize about a possible co-evolution between V. vinifera and GRSPaV.

Subsequently, Pantaleo et al., 2016 analyzed defense response to water stress of asymptomatic grapevines infected with GRSPaV. Results showed that infected plants exhibited higher tolerance to initial levels of water stress and a miRNAs profile
significantly different than healthy plants. In addition, changes in leaf composition, such as a higher cell density and a bigger number of stomas were observed. All these changes could be effects that contribute to plant estress adaptation, which led the authors to support the co-evolution hypothesis and propose a symbiotic mutualism interaction between GRSPaV and asymptomatic grapevines.

**Characteristics of GRSPaV**

GRSPaV belongs to the genus *Foveavirus*, family *Betaflexiviridae*, order *Tymovirales*, which is characterized by containing monopartite and filamentous viruses, lacking in envelope and with a linear genome according to the classification of International Committee on Taxonomy of Virus (ICTV).

At molecular level, the genetic material of this virus is a positive single strand RNA of approximately 8725 nucleotides, characterized by a high variability in its sequence, which has greatly difficulted the development of adequate and efficient detection protocols for GRSPaV (Alabi *et al*, 2010; Hooker, 2017; Meng *et al*, 2006; Terlizzi *et al*, 2010). The little nucleotide similarity found between the 15 different isolates sequenced to date has led to propose 8 phylogenetic groups designated with the name of the first sequenced isolate: GRSPaV-PN, GRSPaV-SY, GRSPaV-1, GRSPaV-SG1, GRSPaV-BS, GRSPaV-ML, GRSPaV-JF y GRSPaV-LSL (Meng & Rowhani, 2017).

Despite the high variability present in the genomic sequence of the virus, all sequenced viral isolates show an identical genomic structure to each other, consisting of 5 open reading frames (ORF1-5) which code for different proteins
necessary for viral replication. Specifically, ORF1 encodes a replication polyprotein containing a methyltransferase (MTR), a helicase (HEL) and a RNA-dependent RNA polymerase (RdRP). ORF1 is followed by a triple gene block (TGB) consisting of ORF2, 3 and 4 that encode three movement proteins that act together to allow GRSPaV movement through the plant. Finally there is ORF5 which encodes GRSPaV capsid protein (CP) (Meng & Rowhani, 2017).

Considering the above, there is a wide variety of primers designed for GRSPaV detection, with different degrees of efficiency depending of strain and fragment of viral sequence to amplify (Alabi et al., 2010; Meng et al., 1999; Terlizzi et al., 2011). However, in recent years, the use of degenerate primers amplifying some of the most conserved regions of viral sequence has allowed detect different GRSPaV variants (Lima et al., 2006; Osman et al., 2008; Terlizzi et al., 2011).

In terms of viral distribution inside the plant, GRSPaV is a phloem-restricted virus, capable of accumulating largely in grapevine berries and possible to detect in fruit and its by-products several years after this has been harvested (Gambino et al., 2012). Furthermore, it is possible to detect the virus efficiently in almost all tissues, including roots and plant stems (Hu et al., 2018).

Efficiency of GRSPaV elimination.

GRSPaV has a high resistance to conventional sanitation techniques (Maliogka et al., 2015). Additionally, the efficiency rate of virus elimination varies significantly depending on technique and cultivar to clean up.
A complete study on the success of conventional techniques for GRSPaV elimination was carried out by Gribaudo et al. (2006). Here, the authors evaluated the efficiency of *in vivo* and *in vitro* thermotherapy, meristem culture and somatic embryogenesis in sixteen different grapevine cultivars of north-east Italy. Results showed that both thermotherapy formats, as well as meristem culture, did not exceed 30% of virus elimination success, while all plants that survived to somatic embryogenesis treatment were free of virus, independent of cultivar analyzed (Gribaudo et al., 2006). However, the long period required to obtain plant material, high plant mortality and high probability of somaclonal variation makes this tool an unprofitable sanitation alternative. Especially because one of the most important characteristics of wine industry is that it pursues a precise and persistent clonal identity in its vineyards.

In order to improve GRSPaV elimination efficiency, subsequent studies have combined thermotherapy and meristem culture. In some works, results showed a significant improvement in sanitation success (Maliogka et al., 2009), however, the results continue to be highly dependent on the cultivar used (Skiada et al., 2012).

Techniques used against viruses that attack animal hosts such as chemotherapy have also been tested against *V. vinifera* pathogens. Compounds such as Tiazofurin (TR), Rivarbin (RB) and Mycophenolic Acid (MPA) were used to eliminate GRSPaV from two cultivars: Agiogitiko and Malagouzia (Skiada et al., 2013). Results showed that the highest rates of virus elimination were achieved for Agiorgitiko cultivar with 10 µg/ml of TR, 30 µg/ml of RB or 20 µg/ml of MPA, the latter being also highly toxic to the plant. However, GRSPaV elimination rates obtained on Malagouzia were about half that reached on Agiorgitiko, indicating that chematherapy success is also
closely related to the cultivar. For this reason, the development of new GRSPaV elimination strategies acquires relevance in the production of virus-free plant material.

In this context, copper-based compounds emerge as an interesting alternative due to their antimicrobial properties, broad spectrum and unespecific mechanism of action (Borkow & Gabbay, 2005; Thurman, Gerba, & Bitton, 1989.), which makes it an excellent option for treatment against viruses with high genomic plasticity, such as GRSPaV.

The antimicrobial properties of copper (Cu) have been extensively studied against pathogens such as bacteria and fungi, however literature related to its antiviral potential is less abundant. Studies performed so far have evaluated the antiviral properties of different copper formats, with variable results. For instance, it has been reported that Cu sheets are capable of affecting the population and infectivity of pathogens such as Influenza A Virus (Noyce et al, 2007) and Murine Norovirus (Warnes et al, 2015), one of the main causal agents of gastroenteritis in humans. In both cases, exposure to copper only required a few hours to achieve a significant reduction in viral population, while its mechanism of action involved not only RNA degradation, but at least partial degradation of capsid protein.

Copper-based compounds have also shown an important viricidal effect. In an article published by Karlstrom & Levine (1991), authors were able to inactivate almost completely the functioning of the Human Immunodeficiency Virus 1 protease, a key enzyme in viral replication. Copper solutions have also been able to inactivate and
eliminate the Influenza A virus (H9N2) present in MDCK cell culture in concentration that was not toxic for its host (Horie et al., 2008). Viricidal properties of copper are not only useful against RNA viruses, but also actively act against viruses whose genetic material is DNA, such as the Herpex Simplex Virus (HSV), pathogen that is efficiently eliminated by Cu$^{+2}$ solutions (Sagripanti, 1997).

It has been seen that action mechanism of Cu requires direct contact with the pathogen. In addition, copper is a cation, and as such, is subjected to ionic diffusion conditions of the plant. Therefore it is necessary to analyze the option of new copper formats with greater contact surface and whose diffusion is not limited by the plant capacity to absorb ions. Nanoparticles of copper (NPs-Cu) arise as an interesting alternative to perform sanitation treatments, even more considering that antiviral properties of this metal format showed a better rate of elimination of some viruses than ionic version of copper (Shionoiri et al., 2012).

Certification program of plant material

The high susceptibility of grapevine to pathogen infection has led the global wine industry to a constant concern state for disease control. In this context, it has been observed that the use of sanitized plant material in vineyard establishment could lead to several years of disease-free fields and the future reduction of pathogen transmission via insects. This, because pathogen load does not reach the same level as in a recently contaminated plant (Gribaudo et al., 2009).

Based on this, during the last decades the European Union has established some measures to improve the quality of the initial plant material (G P Martelli, 1999).
Recent guidelines that mainly regulate the system of production and marketing of healthy vine plants in the European Community have forced other countries involved in the market to develop their own measures of plant material Certification. Since the diseases present in different regions of the world can greatly vary, it is necessary to develop local research to understand the state of infection of the vineyards and the effect they have on vine crops of each region.

Chile currently has certification standards for the propagation material of *Vitis* spp., a process supervised by the Servicio Agrícola y Ganadero. However, informal propagation carried out by producers who take propagation material from already infected vines is a common practice both in Chile as well as in the world. In addition, plant certification process by nurseries is voluntary (Exempt Resolution No.: 7605/2013.2007), and in most cases is not performed, due to its high cost.

Certification of plant material includes confirmation that plants are free of different pathogens including 6 of the main vine-infecting viruses: GLRaV-1, -2 and -3; GFLV, GVA and GVB. However, in Chile as well as in different countries of importance for viticulture, the detection of GRSPaV is not included in the certification process, which allows the sale and transport of plant material contaminated with this virus.

The main reasons for not considering this virus in the grapevine certification process are associated to the fact that it is highly recalcitrant to conventional sanitation, but even more important, there is insufficient evidence of the real impact that this virus has on the performance and useful life of *V. vinifera*. 
Approach to the problem

In view of this situation, the objective of the present work was to deepen the knowledge about the effect of GRSPaV on *V. Vinifera*. Specifically, the effect of simple and mixed GRSPaV-infections on Cabernet Sauvignon cultivar was studied, in order to evaluate whether the presence of GRSPaV modifies growing conditions of the vine in early stages of development. Moreover, it will be analyzed how these results can impact the symbiotic mutualism hypothesis when it is evaluated in early stages of plant development.

In addition, new alternatives for GRSPaV elimination were explored with tools never used for this purpose in the cultivation of vines. For this, the viricidal potential of copper was evaluated, a compound that stands out for a broad spectrum action, using two formats of copper, in order to maximize possibilities for metal diffusion inside the plant and thus provide new alternatives for eliminating GRSPaV, one of the most recalcitrant viruses to conventional sanitation techniques.

General Objective

To study the effect of GRSPaV on growth and gas exchange parameters in *V. vinifera* cultivar Cabernet Sauvignon in plants with simple and mixed viral infections of GRSPaV. Additionally, evaluate new strategies for eliminating GRSPaV using unconventional sanitation techniques.
Specific hypothesis 1

Change induced by GRSPaV in parameters associated with growth and photosynthesis in plants of *Vitis Vinifera* L. cultivar Cabernet Sauvignon, increase when a co-Infection with GFLV is developed.

Specific objectives

1. To compare the effect of GRSPaV on growth among virus-free, GRSPaV infected and GRSPaV- and GFLV-infected plants.

2. To compare the effects of the virus in gas exchange parameters between virus-free, GRSPaV.infected and GRSPaV- and GFLV-infected plants.

3. Evaluation of gene expression levels related to growth processes, gas exchange and defense response in grapevine plants with simple and mixed infections of GRSPaV and GFLV.

Specific hypothesis 2

Grapevines cultivar Cabernet Sauvignon infected with GRSPaV can be sanitized by unconventional techniques of plant material sanitation.

Specific objective

4. To evaluate the elimination rate of GRSPaV in *V. vinifera* Cabernet Sauvignon cultivar treated whit two copper formats.
References


Response of *Vitis vinifera* cultivar Cabernet Sauvignon on Early Stages of Development to simple and mixed infections of *Grapevine rupestris stem pitting associated virus*.

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This chapter was sent to *Horticulture Research* – Nature in March 06, 2019.
Abstract

*Grapevine rupestris stem pitting associated virus* (GRSPaV) is one of the most widely distributed viruses; even so, little is known about its effect on *Vitis vinifera*. To provide new insights, the effects of single and mixed GRSPaV infections on the *V. vinifera* cultivar Cabernet Sauvignon were studied by evaluating growth parameters, such as measurements of the total plant length, the number and distance of internodes and the number of leaves per shoot. In addition, parameters relating to gas exchange, *i.e.*, the stomatal conductance, net photosynthetic rate, internal CO$_2$ concentration and leaf transpiration, were also assessed. All the measurements were performed in one- and two-year-old plants with a single GRSPaV infection or mixed infections of GRSPaV and *Grapevine fanleaf virus* (GFLV). The results show that the plant phytosanitary status did not significantly alter the growth and gas exchange parameters in one-year-old plants. However, in two-year-old plants, single GRSPaV infections increased shoot elongation, which was accompanied by the overexpression of genes associated with the gibberellic acid response pathway. The gas exchange parameters of these plants were negatively affected, despite exhibiting higher *LHCII* gene expression. Plants with mixed infections did not have modified growth parameters, although they presented a greater reduction in the primary photosynthetic parameters evaluated with no change in *LHCII* expression. The results presented here confirm the symbiotic mutualism hypothesis for *V. vinifera* and GRSPaV during the early stages of plant development, and they provide new evidence about the effects of GRSPaV and GFLV co-infections on the Cabernet Sauvignon cultivar.
Introduction

The grapevine (Vitis vinifera spp.) is one of the most important crops in the world. Its economic relevance has positioned it as one of the most studied fruit species in agricultural science, which has allowed researchers to identify approximately 70 different viruses to date that infect this species. The grapevine rupestris stem pitting associated virus (GRSPaV) is one of the most ubiquitous and variable viruses, and it is capable of infecting several species in the Vitis genus. The presence of GRSPaV has been closely related to the development of rupestris stem pitting syndrome, which belongs to the rugose wood grapevine disease complex, as well as the “vein necrosis” disease observed under a Richter-110 indicator, and some other disorders with varying levels of severity. However, sufficient evidence to confirm that this virus is the causal agent of these diseases is still lacking.

Moreover, evidence of the effect of GRSPaV infection on the physiological performance of the plant and their impact of GRSPaV on productivity parameters is also insufficient. Some studies have shown that the presence of this virus in asymptomatic grapevines would not affect the yield or the chemical characteristics of the grape berry in different evaluated cultivars. In some cases, there were differences depending on factors such as the cultivar and the climatic conditions in which the experiment was performed.

A study performed in 2012 in Italy found no effect from GRSPaV on the yields of Bosco grapevines. Additionally, the authors presented a complete analysis of the GRSPaV effect on the physiological parameters of grapevines. The results showed
that infected plants had a lower chlorophyll content in the leaves and a reduced net photosynthetic rate ($P_n$). In this same study, a transcriptomic analysis of the leaves showed that GRSPaV-infected plants presented a higher basal expression of genes associated with the photosynthetic process, such as the genes encoding *rubisco activase (RCA)*, *light harvesting complex I (LHCI)*, and *light harvesting complex II (LHCCI)*, or genes related to carbon fixation such as *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *fructose-bisphosphatase (FBPase)* and *ribulose 1,5 bisphosphate carboxylase (RuBP)*. Another category that displayed an interesting behaviour was the stress response gene group, in which most overexpressed genes belonged to the abiotic stress response group while most of the repressed genes fit in the biotic stress response classification. These results led the authors to hypothesize about the possible beneficial effect of the virus, which would produce a basal over-expression of the response against abiotic factors.

Subsequent studies have addressed the response of GRSPaV-infected plants under water stress, finding that individuals with latent GRSPaV infections have a differential profile in their miRNAs expression that allowed the plants to have a greater resilience to initial levels of water stress. This expression was accompanied by modifications in some eco-physiological parameters such as an increased cell density and stomata number, which would confirm a close plant-virus co-evolution. These results lead the authors to propose a symbiotic mutualistic relationship between GRSPaV and *V. vinifera*, in which the presence of the virus would confer a greater capacity to cope with the initial levels of abiotic stress to the plant, among other traits, due to the basal induction of genes related to the abiotic stress response. This approach would confer
an adaptive advantage to the plant; however, the transcriptomic analyses performed by Gambino et al.\textsuperscript{9} also showed the basal repression of a large number of genes associated with the defence response against biotic stresses, an issue that, in our opinion, has not been sufficiently explored and that could modify this hypothesis.

Considering all the above information, the aim of the present study was to provide new evidence regarding the effect of GRSPaV on \textit{V. vinifera}, specifically on cv. Cabernet Sauvignon, in relation to the gas exchange processes. The aims were also to address the effect of this virus on the molecular and phenotypic growth parameters of plants with single GRSPaV infections and plants co-infected with GRSPaV and the \textit{grapevine fanleaf virus} (GFLV).

\textbf{Results}

To evaluate the possible effects of GRSPaV on grapevines with single and mixed infections, measurements of the growth and photosynthetic parameters and the expression levels of the genes involved in these processes were performed.

\textit{Growth monitoring in plants with single and mixed GRSPaV infections}

The effect of GRSPaV on plants with single and mixed viral infections was studied by performing growth monitoring on Cabernet Sauvignon grapevines with three different phytosanitary statuses: virus-free, GRSPaV-infected and GRSPaV- and GFLV-infected plants.

The total shoot length results are shown in Figure 1. One-year-old plants with different viral infections have similar shoot elongation results relative to virus-free
Figure 1. Monthly monitoring of shoot elongation in 1-year and 2-year-old plants. The monthly growth was measured in 1 (a) and 2-year-old plants (b) with three phytosanitary conditions: virus-free, infected with GRSPaV and infected with GRSPaV and GFLV during the 2017-2018 growth period. The data and include three to eight replicates and were analysed by ANOVA, and the graphs show the mean ± SE.
plants throughout all the seasons (Figure 1a). However, in two-year-old plants, a
difference in the shoot elongation was observed since very early in the season; the
GRSPaV-infected plants exhibited significantly higher shoot elongation than the
control (Figure 1b). In addition, it is possible to observe that healthy plants and plants
with mixed infections displayed the same total shoot elongation, statistically, in both
one- and two-year-old grapevines.

The second growth parameter evaluated here was the average internode length in
one- and two-year-old plants. In the first case, no differences were observed in the
average lengths of the internodes regardless of the phytosanitary status during the
season, except during the first month of evaluations, when significantly higher
magnitudes were observed in plants with mixed infections in comparison to the other
conditions (Figure 2a). The results from the two-year-old plants showed that the
individuals with simple GRSPaV infections exhibited a significantly longer average
internode length than the other conditions evaluated here. This result was consistent
throughout the season (Figure 2b).

When the number of internodes throughout the shoot was observed (Figure 2c and
d), the results were similar to those obtained for the previously described
parameters. In one-year-old plants, there were no differences in the internode
numbers when comparing the different phytosanitary conditions (Figure 2c). However, in two-year-old plants, the grapevines infected only with GRSPaV
presented a greater number of internodes than the other two groups of evaluated
plants (Figure 2d). In addition, there were no observed differences when comparing
the number of internodes in virus-free plants and plants with mixed viral infections.
Figure 2. Monthly monitoring of growth parameters in 1-year and 2-year-old plants. The average lengths of the internodes (a and b), the number of internodes (c and d) and the number of leaves per shoot (e and f) were measured in 1 (a, c and e) and 2-year-old plants (b, d and f) under three phytosanitary conditions: virus-free, infected with GRSPaV and infected with GRSPaV and GFLV. The data and include three to eight replicates and were analysed by ANOVA, and the graphs show the mean + SE.
Regarding the number of leaves per shoot (Figure 2e and f), the results were similar to the previously described ones, although at the end of the season, a greater number of leaves was observed in virus-free grapevines from one-year-old plants in comparison to the other phytosanitary conditions. In two-year-old plants, the group with a significantly higher number of leaves was the group of plants infected with GRSPaV (Figure 2f).

To establish any type of correspondence between these results and the viral load, the GRSPaV and GFLV concentrations were quantified in samples collected in both January and March from one-year and two-year-old plants. The results show that there were no statistically significant differences in the viral loads of the samples under any of the conditions or ages under analysis (Supplementary Figure 2).

**Evaluating gas exchange parameters in plants with single and mixed infections of GRSPaV**

Figure 3 shows the results obtained for the gas exchange parameters evaluated here (the $P_n$, $C_i$, $g_s$ and $E$). The $P_n$ levels in one-year-old plants exhibited no differences between the three different phytosanitary statuses; however, in two-year-old plants, those with mixed infections yielded significantly lower $P_n$ values. When the $C_i$ was measured in one-year-old plants, a significant reduction of this parameter was observed in GRSPaV-infected plants, whereas in two-year-old plants, the group with double viral infections showed a higher $C_i$ value. The $g_s$ and $E$ results did not show significant differences when comparing the different phytosanitary statuses or plant ages.
Figure 3. Measurements of gas exchange parameters. The net photosynthesis rate (Pn) (a and b), internal concentration of leaf CO\(_2\) (Ci) (c and d), stomatal conductance (gs) (e and f) and transpiration rate of the leaf (E) (g and h) in 1-year-old (a, c, e and g) and 2-year-old plants (b, b, f and h) with three phytosanitary conditions: virus-free, infected with GRSPaV and infected with GRSPaV and GFLV. The data include three technical repetitions and were analysed by ANOVA and the graphs show the mean \( \pm \) SE.
**Quantifying levels of gene expression in plants with single and mixed GRSPaV infections** To study if simple and mixed GRSPaV infections can affect the expression of genes involved in metabolic pathways that are directly related to the growth, photosynthetic processes and defence response of the plant, an expression level quantification was performed for selected genes.

In relation to the plant growth, the genes involved in the gibberellic acid (GA) response pathway were selected. Figure 4 shows the relative expression levels of genes encoding some of the most important regulators of GA pathways, namely **DELLA1**, **GID1b** and **SLY1a** (Figure 4a, b and c, respectively). In addition, the expression of the following genes downstream from the GA signal was evaluated: **GASA1**, **GASA3** and **GASA6** (Figure 4d, e and f, respectively).

For **DELLA1** (Figure a), the observed differences were not significant for any of the comparisons, and the gene expression levels presented a high standard deviation. The expression levels of the **GID1b** gene (Figure 4b) were higher in samples collected in March from one-year-old plants infected with GRSPaV in comparison to the other phytosanitary conditions. This difference was not repeated for the other evaluated time points, although the trend was maintained in March for two-year-old plants, with gene expression levels that were significantly lower than those of the one-year-old plants (Supplementary Table 2). The same behaviour was observed in the expression levels of **SLY1a** (Figure 4c). Finally, the two-way ANOVA shows that the **SLY1a** expression levels during March in one-year-old plants were significantly higher than the levels quantified in the two-year-old plants (Supplementary Table 3).
Figure 4. Relative expression of genes associated with the GA response pathway. The relative expression of regulator genes of the GA response, *DELLA1* (a), *GID1b* (b) and *SLY1a* (c), was evaluated. Additionally, the relative expression of genes downstream of the GA stimulus was evaluated for *GASA1* (d), *GASA3* (e) and *GASA6* (f). The graphed expression levels are presented relative to the reference gene, ubiquitin and include three biological replicates and two technical repetitions. The data were analysed by two-way ANOVA to compare the variances between phytosanitary conditions and between different plants ages. The graphs show the mean ± SE.
In evaluations on the expression of genes downstream from the GA signal, *GASA1* was significantly more highly expressed in GRSPaV-infected plants. This trend was observed during January and March in one-year-old plants and in January in two-year-old plants (Figure 4d). Additionally, the two-way ANOVA shows that there was a higher transcript level in the two-year-old plants when comparing samples between the differently aged plants collected in January. The same results were obtained when comparing the expression levels of *GASA1* between plants of different ages in March (Supplementary Table 4). For *GASA3*, higher gene expression was observed in one-year-old plants infected with GRSPaV during March (Figure 4e). For *GASA6*, the gene was overexpressed in GRSPaV-infected plants at most of the analysed time points except for March, in the two-year-old plants (Figure 4f). The two-way ANOVA shows that in a comparison of *GASA6* expression levels in samples collected in January, this gene was significantly more highly expressed in two-year-old plants. No differences were found between the samples collected in March from differently aged plants (Supplementary Table 6).

The next measurements to be performed were the relative quantifications of genes related to the photosynthetic process (*LHCII*) and chlorophyll degradation (*ACD1*) (Figure 5). For *LHCII* (Figure 5a), higher gene expression was observed in GRSPaV-infected plants with respect to the other evaluated conditions, although the most significant difference was only in March for one-year-old plants and in January for two-year-old plants. For *ACD1* (Figure 5b), a similar result was observed in one-year-old plants, and there were no differences in the *ACD1* expression when comparing different phytosanitary statuses within the two-year-old plant group.
Figure 5. Relative expression of genes involved in photosynthetic processes.
The relative expression of *LHCII* (a) and *ACD1* (b) was evaluated. The graphed expression levels are presented in relation to the reference gene, ubiquitin and include three biological replicates and two technical repetitions. The data were analysed by two-way ANOVA to compare the variances between phytosanitary conditions and between different plant ages. The graphs show the mean ± SE.
Additionally, the two-way ANOVA shows that one-year-old plants have greater levels of ACD1 expression than two-year-old plants (Supplementary Table 8).

Finally, the PAL and CAT3 gene expression was measured, and both genes were involved in the secondary metabolism of the grapevine (Figure 6). The PAL expression was greater in two-year-old plants with simple GRSPaV infections in both January and March (Figure 6a). A similar expression profile was observed in one-year-old plants, but these differences were not significant (Supplementary Table 9). Regarding the CAT3 levels, one-year-old plants infected with GRSPaV showed higher expression of this gene in March, and higher average CAT3 expression was observed in samples collected in January from one- and two-year-old plants (Figure 6b). No differences in the CAT3 expression were observed for any of the other phytosanitary statuses.

**Discussion**

The growth and photosynthetic parameters were measured in one- and two-year-old plants with different phytosanitary statuses. When the growth parameters were studied in the one-year-old plants (Figure 1 and 2), no differences were observed between the groups under different phytosanitary conditions, whereas in the two-year-old plants, greater vigour was observed in GRSPaV-infected grapevines, which was explained by their greater number of leaves per shoot as well as the greater elongation of the main shoot, a product of a greater number and length in the internodes. These results differ from other previously reported results in different cultivars, in which GRSPaV was found to have minimal or no effect on the vigour of
Figure 6. Relative expression of genes involved in secondary metabolism. The relative expression of *PAL* (a) and *CAT3* (b) was evaluated. The graphed expression levels are presented relative to the reference gene, ubiquitin and include three biological replicates and two technical repetitions. The data were analysed by two-way ANOVA to compare the variances between the phytosanitary conditions and between different plant ages. The graphs show the mean \(\pm\) SE.
Albano\textsuperscript{11}, Madeleine Sylvaner, Ortega\textsuperscript{8} and Savagnin rose\textsuperscript{12} cultivars. However, all of those studies measured the vigour as the average pruning weight and were mostly performed under field conditions in five- to eight-year-old plants, so these and other experimental differences make it difficult to establish a comparison.

Regarding the growth results in plants infected with GRSPaV and GFLV, this group of plants did not show significant differences in relation to virus-free plants. These results are unexpected since there are important negative effects from the GFLV virus on the plant vigour\textsuperscript{7} and total shoot length\textsuperscript{13}. However, in those papers, no GRSPaV detection analyses were reported, and the authors did not mention anything about a possible co-infection with GRSPaV, a virus that is almost impossible to detect without molecular analyses; this makes it difficult to establish a comparison with the results shown here for plants with mixed infections. Furthermore, reduced plant growth is frequently associated with the development of typical fanleaf disease symptoms. By contrast, the results presented here were evaluated in asymptomatic plants that did not present fanleaf disease, since the ones that began to show symptoms developed accelerated decay and death, therefore we could not incorporate their data into the results (data not shown).

Regarding the photosynthesis measurements, the $P_n$ levels in one-year-old plants did not show significant differences between the different phytosanitary conditions, although plants with simple GRSPaV infections tend to exhibit higher values for this parameter. By contrast, it is possible to appreciate a tendency towards reduced $P_n$ values in two-year-old plants as their phytosanitary status worsens, for a significant reduction in the $P_n$ of two-year-old plants with double infections. The relatively low
Pn values obtained in this study probably occurred because the measurements were performed on plants growing in greenhouses. The Pn values are concordant with the Ci results obtained here, since the plants that showed higher Pn levels have significantly lower Ci values. This trend in the behaviour of photosynthetic parameters is similar to the one observed by Gambino et al.9.

Finally, no significant differences were observed in the gs and E, independent of the phytosanitary status, which confirms the previous evidence that the photosynthetic reduction caused by viral infections is not a consequence of stomatal closure in leaves9,14.

It is interesting to note that in the case of photosynthetic measurements, plants with mixed viral infections presented a more severe alteration with respect to the simple GRSPaV-infected and virus-free plants, contrary to what was observed in the growth measurements in Figures 1 and 2. Therefore, the presence of GRSPaV and GFLV viral infections in one- and two-year-old plants would reduce the ability to fix CO2 without significantly reducing the shoot elongation during the early stages of V. vinifera development. However, with these results, it would be expected that the vigour of the co-infected plants would be affected during the stages of grapevine development to follow as reported in the literature.

The first studied genes were some of the primary regulators of the GA pathway, a plant hormonal route associated with plant growth and elongation15,16. It was possible to observe that for DELLA1, a basal inhibitor of the GA response pathway, although the mean gene expression is higher in GRSPaV-infected plants, the
variability of the data did not allow for the establishment of significant differences between the three analysed groups. Additionally, repressors of *DELLA1*, *GID1b* and *SLY1a*, and one of the genes activated downstream of the GA signal, *GASA3*, show significantly higher expression in samples collected in March from one-year-old plants infected with GRSPaV in relation to the control group. *GASA1* and *GASA6* also showed greater expression in GRSPaV-infected plants, but it is also possible to observe this difference in January in one- and two-year-old plants. This basal overexpression of *GID1b* and *SLY1a* could indicate the possibility of a higher *DELLA1* degradation rate, which could influence the sensitivity of the plant to GA and would explain, at least partially, the overexpression of GA response genes, such as *GASA1*, 3 and 6, in plants infected with GRSPaV. It is interesting to note that at the end of the summer, two-year-old plants infected with GRSPaV consistently exhibited a basal expression of the three evaluated genes. The lower expression level of these genes could involve a reduction in the GRSPaV effect due, for instance, to reduced virus replication or another issue that we have not considered in this study. Additional studies are necessary for a better comprehension of these results.

Plants with double viral infections did not show differences in the expression levels of GA response genes in comparison with virus-free plants, which is consistent with the plant growth results described above. Therefore, it is possible that part of the differences observed in the evaluated growth parameters may be explained by the increased levels of GA response genes, which are cancelled when the GRSPaV infection is accompanied by GFLV infection.
The changes in the expression of genes related to photosynthetic processes (LHCII and ACD1) were consistent with those reported by Gambino and collaborators\(^9\), which would support the basal overexpression of these genes in GRSPaV-infected plants. Additionally, in a comparison of virus-free plants and plants co-infected with GRSPaV and GFLV, there were no significant differences in the expression levels of photosynthetic genes. Therefore, the overexpression of LHCII and ACD1 produced by GRSPaV is lost when there is a co-infection with GFLV, which could explain the detrimental effect of the double infection on the measured photosynthetic parameters. It is interesting to observe that the average expression of ACD1 in double-infected plants is higher than its expression in virus-free plants. This finding may have occurred because the GFLV replicative mechanism leads to greater chlorophyll degradation, which could explain the increased ACD1 expression in comparison to the virus-free plants.

Finally, the quantification of PAL, a precursor for the phenylpropanoid pathway, and therefore a variety of secondary metabolite associated with defence processes, growth and plant development\(^{17}\), showed an expression profile similar to the aforementioned genes, in which GRSPaV-infected plants exhibited higher levels of gene expression than the other groups of plants. Although it is interesting to highlight that the greatest increase in PAL expression was observed in two-year-old plants infected with GRSPaV, unlike most of the previous results, in which increased gene expression was registered in one-year-old plants. Increased PAL expression has also been observed in response to other viral infections such as the GLRaV-3 infection\(^{18}\) as well as other pathogens, such as Plasmopora viticola \(^{19}\), or the
nematode *Xiphinema index*\(^{20}\), which is a vector of GFLV. By contrast, the expression of *CAT3*, the gene associated with cell detoxification induced by stress, is higher in one-year-old plants. Double-infected plants showed higher average *CAT3* expression in most cases than in virus-free plants, consistent with other reports, which is also congruent with the results obtained for the *ACD1* gene, another gene related to cell detoxification.

In summary, the results submitted here allow us to provide new evidence about the effect of GRSPaV on one- and two-year-old *V. vinifera* plants. Specifically, our findings indicate that the presence of GRSPaV would have a positive effect on different growth parameters and shoot elongation caused in conjunction with the increased activation of genes involved in the GA response pathway, despite showing a lower net photosynthetic rate and lower CO\(_2\) assimilation. Plants with simple GRSPaV infections also exhibited an overexpression of genes related to photosynthetic and cell detoxification processes.

In addition, the double infection with GRSPaV and GFLV did not affect the shoot elongation during early stages of plant growth, although the damage caused to the gas exchange parameters at this stage of grapevine development could modify these results during future seasons and could lead to the development of the typical symptoms caused by GFLV.

These results conform to the hypothesis on the symbiotic mutualistic interaction between GRSPaV and *V. vinifera* proposed by Pantaleo *et al.*\(^{10}\), and they confirm a
beneficial effect from GRSPaV on the early stages of grapevines development in the Cabernet Sauvignon cultivar.

From an agronomic point of view, these data confirm the scarce possibility of visually evaluating the presence of GRSPaV and especially GRSPaV and GFLV in mixed infections, in plants acquired by producers for vineyard establishment. It is only through the application of a sampling protocol and the performance of laboratory tests that it is possible to ascertain the health status of the plant material.

**Materials and methods**

Evaluations of the growth and physiological parameters and the quantification of the expression levels of genes involved in the processes of interest were performed in plants with three different phytosanitary conditions.

**Plant Material**

*Vitis vinifera* plants from the Cabernet Sauvignon cultivar were analysed by qPCR detection for the 8 most important viruses, namely GLRaV-1, -2 and -3; GVA, GVB, GFLV, GRSPaV and GFkV. Plants with three different phytosanitary statuses were selected: virus-free, GRSPaV-infected and GRSPaV- and GFLV-infected plants.

The selected grapevines were multiplied *in vitro* and then acclimated during the spring of 2016 (two-year-old plants) and 2017 (one-year-old plants). After that, the plants were established in 3-L pots in greenhouses located at the San Joaquín Campus of Pontificia Universidad Católica de Chile (Santiago, Chile). The pots were
arranged in a completely random design consisting of three to eight biological replicates per phytosanitary status per plant age.

**Growth measurements**

The monitoring of the growth of plants under different phytosanitary conditions was performed by evaluating the following parameters: the shoot elongation, number and length of the internodes and the number of leaves per shoot. To facilitate a comparison between replicates, the plants were managed such that the growth of a single main shoot was allowed, and the growth of lateral shoots was prevented.

Growth measurements were performed in the one-year-old plants using 6 to 8 biological replicates per phytosanitary status. For the two-year-old plants, the measurements included 3 to 6 replicates per group.

The evaluations were performed between October 2017 and March 2018 for the two-year-old plants and between November 2017 and March 2018 for the one-year-old grapevines. During this time, the parameters mentioned above were measured every 4 weeks.

**Evaluating gas exchange parameters**

During January of 2018, the following parameters were evaluated: the photosynthetic net rate (P\textsubscript{n}), leaf internal CO\textsubscript{2} concentration (C\textsubscript{i}), stomatal conductance (g\textsubscript{s}) and leaf transpiration rate (E). For these measurements, an infrared gas analyser (IRGA) Handheld Photosynthesis System model CI-340 (CID BIO-Science, Inc.) was used.
For optimized measurement, the plants were removed from the greenhouses and evaluated outdoors between 10 a.m. and 1 p.m., and the measurements were performed using mature leaves. Between 3 and 7 biological replicates were used for each phytosanitary condition. The resulting data were analysed using analysis of variance (ANOVA).

**RNA extraction and quantification of relative expression levels**

The leaf samples were collected in January and March of 2018 from three different groups of plants. The samples were frozen in liquid nitrogen and stored at -80°C.

RNA was extracted using a 3% CTAB protocol modified from Yu and collaborators\(^{22}\). The quantity and quality of the extracted RNA were determined using both fluorometer (Qubit 4, Thermo Fisher Scientific) and Nanodrop (Nanodrop 2000, Thermo Fisher Scientific) instruments. The cDNA synthesis was performed with an Affinity Script QPCR cDNA synthesis kit (Agilent Technologies) according the manufacturer’s instructions, beginning with 0.5 μg of RNA. Real-time PCR was performed using 2 μL of cDNA and Brilliant II SYBR® Green QPCR Master mix (Agilent Technologies), with an Mx3000P detection system (Stratagene). The primer sequences used here were obtained from the literature as follows: \(ACT^{23}\), \(UBQ^{24}\), \(DELLA1\), \(DELLA3\), \(Gid1b\) and \(SLY1a^{25}\), \(GASA1\), \(GASA3\) and \(GASA6^{26}\), \(LHCII\), \(ACD1\), \(PAL\) and \(CAT^{9}\) and \(GAPDH^{27}\). The qPCR conditions were: 95°C for 10 min for initial denaturation, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 sec and a final extension at 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. The gene expression levels were normalized using the reference genes ubiquitin.
(UBQ) and actin (ACT). The relative expression of the evaluated genes was calculated using the comparative C\textsubscript{t} method ($2^{-\Delta\Delta C\text{t}}$)\textsuperscript{28} with three biological replicates and two technical replicates. The specificity of reaction was monitored by evaluating the dissociative curves at the end of every qPCR. The gene expression was calculated and graphed as the mean and standard deviation.

Additionally, the concentrations of GRSPaV and GFLV were quantified in the same samples. The qPCR mix consisted of 2 µL of 10X buffer, 1 µL of MgCl\textsubscript{2} 50 mM, 0,75 µL of dNTPs 10 mM, 14,3 µL of nuclease-free water, 0,2 µL of Platinum Taq DNA Polymerase (Invitrogen) and 0,75 µL of primer mix. The primer sequences used here were obtained from the literature\textsuperscript{21}.

**Statistical analyses**

The results for the growth monitoring, physiological parameters and gene expression levels collected from plants of the same ages and different phytosanitary statuses were analysed by one-way ANOVA. Additionally, multifactorial comparisons of the gene expression levels were performed between plants with different ages and equal phytosanitary statuses by two-way ANOVA. In both cases, a Fisher’s mean comparison was performed and the significant differences between the means were assigned considering values of $p < 0,005$. 
ACKNOWLEDGEMENTS

Mariola Tobar acknowledges funding by Comisión Nacional de Ciencia y Tecnología (CONICYT), folio scholarship 21141168. We are grateful to I+D Vinos de Chile (Project 12CTI-16788-01) for provide the plant material.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

CONTRIBUTIONS

M. T. planned and designed the research, performed most of the experiments, analysed the data and wrote the article; N. F. contributed to viral detection and revised the article; A. P. critically revised the manuscript and complemented the writing; M. R. revised the manuscript and M. G. supervised all the experiments, complemented and revised the article. All authors read and approved the manuscript.
References


Supplementary Figures

Temperature and humidity conditions in the greenhouses.

**Supplementary Figure 1:** Temperature and humidity conditions in the greenhouses. The plants were grown in greenhouses under controlled temperature and humidity conditions. The average temperature at which the plants grew corresponded to $21^\circ C \pm 3^\circ C$ (A), while the average environmental relative humidity during the measurement season was $60\% \text{ RH} \pm 10\%$ (B).
Supplementary Figure 2: Quantification of viral load. The viral concentration quantifications of GRSPaV (A) and GFLV (B) were performed in January and March in samples of virus-free, infected with GRSPaV and infected with GRSPaV and GFLV plants from 1- and 2-year-old grapevines. A two-way ANOVA was performed to compare the viral load levels between phytosanitary statuses and plant ages. The graphs show the mean ± SE.
**Supplementary Tables**

**Supplementary Table 1:** Effect of the phytosanitary status and plant age on the *DELLA1* expression.

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Means followed by different letters indicate significant differences, while those marked with the initials “ns” indicate that there were no significant differences, considering $p < 0.05$ and using Fisher's comparison test.
**Supplementary Table 2:** Effect of the phytosanitary status and plant age on *GID1b* expression.

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Means followed by different letters indicate significant differences, while those marked with the initials “ns” indicate that there were no significant differences, considering \( p < 0.05 \) and using a Fisher comparison test.
Supplementary Table 3: Effect of the phytosanitary status and plant age on *SLY1a* expression.

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**Supplementary Table 4:** Effect of the phytosanitary status and plant age on \( GASA1 \) expression.

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Means followed by different letters indicate significant differences, while those marked with the initials “ns” showed no significant differences, considering \( p < 0.05 \) and using a Fisher comparison test.
**Supplementary Table 5**: Effect of the phytosanitary status and plant age on *GASA3* expression.

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<td>2</td>
<td>0.396</td>
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<tr>
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<td>12</td>
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</table>

Means followed by different letters indicate significant differences, while those marked with the initials “ns” had no significant differences, considering p < 0.05 and using a Fisher comparison test.
**Supplementary Table 6:** Effect of the phytosanitary status and plant age on *GASA6* expression.

<table>
<thead>
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<th>Age of plant (expression levels)</th>
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<td></td>
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<td>March</td>
<td></td>
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<tr>
<td>1 year</td>
<td>0.1214 A</td>
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<td>2 year</td>
<td>0.1767 B</td>
<td>0.0928 A</td>
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</tr>
</thead>
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<td>Age of Plant</td>
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</table>

Means followed by different letters indicate significant differences, while those marked with the initials “ns” indicate that there were no significant differences, considering p < 0.05 and using a Fisher comparison test.
Supplementary Table 7: Effect of the phytosanitary status and plant age on *LHCII* expression.

LHCII expression

<table>
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<tbody>
<tr>
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<td>January</td>
<td>March</td>
<td></td>
</tr>
<tr>
<td>1 year</td>
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<td>0.1960 A</td>
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ANOVA

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<th>df</th>
<th><em>P</em>-value</th>
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<td>0.052</td>
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<td>Age of Plant x Phytosanitary status</td>
<td>2</td>
<td>0.848</td>
<td>0.195</td>
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<tr>
<td>Residual</td>
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</table>

Means followed by different letters indicate significant differences, while those marked with the initials “ns” indicate that there were no significant differences, considering *p* < 0.05 and using a Fisher comparison test.
Supplementary Table 8: Effect of the phytosanitary status and plant age on ACD1 expression.

<table>
<thead>
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<th></th>
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<tr>
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<td>0.0116 B</td>
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<td>0.0059 A</td>
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ANOVA

<table>
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</thead>
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<td>Phytosanitary Status</td>
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<tr>
<td>Age of Plant x Phytosanitary status</td>
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<tr>
<td>Residual</td>
<td>12</td>
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</tbody>
</table>

Means followed by different letters indicate significant differences, while those marked with the initials “ns” showed no significant differences, considering p < 0.05 and using a Fisher comparison test.
## Supplementary Table 9: Effect of the phytosanitary status and plant age on PAL expression.

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<tr>
<td>1 year</td>
<td>0.00066 ns</td>
<td>0.00027 A</td>
<td></td>
</tr>
<tr>
<td>2 year</td>
<td>0.00056</td>
<td>0.00116 B</td>
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</table>

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>df</th>
<th></th>
<th>P-value</th>
</tr>
</thead>
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<td>0.01</td>
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<td>0.075</td>
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<tr>
<td>Residual</td>
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</tbody>
</table>

Means followed by different letters indicate significant differences, while those marked with the initials “ns” showed no significant differences, considering p < 0.05 and using a Fisher comparison test.
**Supplementary Table 10:** Effect of the phytosanitary status and plant age on CAT3 expression.

<table>
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<th>df</th>
<th>P-value</th>
</tr>
</thead>
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<td>1 year</td>
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<td>January</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 year</td>
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<td>March</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00235 ns</td>
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<td>0.992</td>
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<td>&lt;0.001</td>
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<tr>
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<td>0.00284 A</td>
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<td>0.152</td>
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<td>0.417</td>
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Means followed with different letters indicate significant differences, while those marked with the initials “ns” showed no significant differences, considering p < 0.05 and using a Fisher comparison test.
Chapter 3

Comparative study of two copper formats as a sanitation technique on *Vitis vinifera* cv. Cabernet Sauvignon.

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\(^2\) Universidad de Chile, Facultad de Ciencias Agronómicas, Avenida Santa Rosa 11315, La Pintana, Santiago, Chile, 8820808.

**Key words:** Santitation, NPs-Cu, GRSPaV, *Vitis*. 
Abstract

Grapevine rupestris stem pitting (GRSPaV) is grapevine virus transmitted mainly through contaminated plant material. In addition, GRSPaV is highly recalcitrant to the conventional sanitation techniques and its elimination rate varies depending on the cultivar treated. In this study, a viral elimination protocol based on two copper formats was developed and evaluated. For this purpose, the growth medium of in vitro plantlets of Vitis vinifera cv. Cabernet Sauvignon was supplemented with increasing concentrations of CuSO$_4$ and copper nanoparticles. Plant development parameters were measured to evaluate the stress level caused by the treatments. The results showed that CuSO$_4$ treatments reduced the fresh total weight, shoot and root length and the number of lateral roots per main root. In contrast, copper nanoparticles treatment did not modify none of the plant development parameters evaluated. Based on these results, the following copper concentration were selected for the viral eliminations test: 50 µM of Cu for the CuSO$_4$ treatments and 40 µM of Cu for the copper nanoparticles. The sanitation test with copper treatments showed that the metal did not eliminate GRSPaV on the evaluated conditions. Despite this, the results published here are the first report of the copper nanoparticles effect on the Vitis vinifera plantlets cv. Cabernet Sauvignon in a in vitro system.
Introduction

_Grapevine Rupestris Stem Pitting associated Virus_ (GRSPaV) is able to infect different species of genus _Vitis_. This virus is potentially the causal agent of rupestris stem pitting syndrome, belongs to the Rugose Wood Complex (Martelli, 2014) and Vein necrosis diseases (Bouyahia _et al._, 2005).

So far, no vector insects of GRSPaV have been identified and its transmission occurs mainly by the use of contaminated plant material (Martelli, 2014), giving rise to a high dissemination rate in all the wine-growing countries of the world.

In addition, GRSPaV is one of the most recalcitrant viruses to conventional sanitation techniques such as meristem culture and thermotherapy. Low GRSPaV elimination rates have been obtained with both techniques (Gribaudo _et al._, 2006) compared to the sanitation results achieved in the elimination of other grapevine viruses (Díaz-Barrita _et al._, 2008; Salami _et al._, 2009; Valero _et al._, 2003). Moreover, the efficiency of these techniques varies depending on the cultivar treated, complicating the possibility of applying these protocols on a large scale (Gribaudo _et al._, 2006; Maliogka _et al._, 2009; Panattoni & Triolo, 2010; Skiada _et al._, 2013). Consequently, the search for new sanitation strategies is positioned as an important area in the field of production of virus-free plant material.

In this context, antiviral compounds used in animal organisms arise as an interesting alternative to explore. One of the most common antimicrobial compounds knowing is copper (Cu). This element has been amply used against a wide range of fungi and bacteria because its broad spectrum of action by a nucleic acid and lipidic membrane
degradation (Borkow & Gabbay, 2005; Thurman, Gerba, & Bitton, 1989.). These characteristics make copper an excellent option against pathogen with a high genomic plasticity as GRSPaV.

Considering that Cu action mechanism requires direct contact between the metal and the pathogen, the first publications evaluated viricidal properties of metallic Cu sheets against microorganisms as Influence A Virus (Noyce et al, 2007) and Murine Norovirus (Warnes et al, 2015), one of the main causal agents of human gastroenteritis. Results showed that Cu sheets reduced population and infectivity of the viruses after a few hours of metal exposition through degradation of viral RNAs and capsid protein.

Subsequent publications evaluated antiviral properties of Cu$^{+2}$ solutions against different RNA viruses as VIH-1 or Influence A Virus (H9N2). In all cases, the treatments inactivated the virus and reduced significantly viral population, either by direct exposure of virus to Cu$^{+2}$ solution (Karlstrom & Levine, 1991) or treating cells infected with non-toxic metal concentrations for the host (Horie et al., 2008). Similar results were exhibited when Cu was used against DNA virus as Herpes Simplex Virus (HSV) (Sagripanti, 1997). Therefore, the use of Cu for elimination of grapevine viruses could be a viable tool for plant material sanitation.

The mobilization of Cu as cation will be subject to the ionic diffusion conditions of the plant. Therefore, it is necessary to consider new Cu formats that have a greater contact surface and whose diffusion is not limited by the ionic transport of grapevine.
Nanoparticles of Cu (NPs-Cu) are metallic particles ranging from 1-100 nm and which are characterized by having a high contact surface in a small particle size (Ingle et al., 2014). This increase the interaction probability of nanoparticles with microbes and potentiates the antiviral properties of Cu (Shionoiri et al., 2012).

Studies about Cu ability to attack viruses infecting plants are almost non-existent (Ingle et al, 2014), even more so when it comes to using NPs-Cu as a tool for plant material sanitation.

In this publication we present a novel study where the potential of treatments with two formats of Cu (CuSO₄ and NPs-Cu) were evaluated as a new strategy for grapevine sanitation. Also, the effect of supplementing growth medium with increasing concentration of CuSO₄ and NPs-Cu on the development of in vitro plants was evaluated

**Materials and methods**

**Plant Material**

The study was performed using *Vitis vinifera* cv. Cabernet Sauvignon grown in vitro with two phytosanitary status: virus-free and GRSPaV-infected. Plants were multiplied in solidified basal Murashine &Skoog (MS) medium and grown in growth chamber at 22 ± 1 °C with a 16 hours photoperiod.

The phytosanitary status was confirmed by qPCR detection of eight of the most important grapevines viruses (GRSPaV, GFLV, GFkV, GLRaV-1, -2 and -3, GVA and GVB).
**Setting copper concentration**

To evaluate copper efficiency as an antiviral agent, this compound was added to the basal medium MS in two formats: CusSO$_4$ and NPs-Cu (Sigma Aldrich).

In order to define the appropriate concentrations for the treatment, an evaluation of different copper concentrations on plantlets development was performed. The following copper concentrations were added to the medium: 0, 5, 15, 50, 75 and 100 uM of CuSO$_4$ and 0, 5, 15, 25, 35 and 45 uM of NPs-Cu. Twenty-four replicates were used per treatment. The plantlets remained for eight weeks in *in vitro* growth chamber and once standardization was completed the following parameters were measured: fresh weight, shoot elongation, number of shoots per plantlets, number of leaves per shoot, length of main root, number of main roots per plantlet and number of lateral roots per main root. Also, foliar samples were taken for copper quantification analysis, which was performed in the Laboratorio de servicios Agroanalisis UC.

In addition, a damage scale with five levels was performed. The description of each level is the following: 1- Shoot growth to the top of the tube. No stress symptoms were observed. 2- Growth reduction of approximately 33%. No stress symptoms were observed. 3- Growth reduction of 33-50%. Slight pigmentation or discoloration on the leaves was observed. 4- Growth reduction of 50% or more. Severe stress symptoms were observed. 5- Dead plants with severe stress symptoms.

The data were analyzed using analysis of variance (ANOVA). The significant differences were assigned considering p<0.05.
**Copper treatments**

_Treatments with CuSO$_4$:_ Twenty four plantlets of _V.vinifera_ cv. Cabernet Sauvignon infected with GRSPaV were stablished in MS medium supplemented with 50 and 65 uM of CuSO$_4$.

_Treatments with NPs-Cu:_ Twenty four plantlets of _V.vinifera_ cv. Cabernet Sauvignon infected with GRSPaV were stablished in MS medium supplemented with 40 and 45 uM of NPs-Cu.

Treated plants remained eight weeks in _in vitro_ growth chamber and after this period the shoot tips of survived plants were transferred to basal medium for an additional eight weeks. Once finished this time, foliar samples were taken and frozen at -80°C for later virus detection analysis.

**RNA extraction and viral detection.**

RNA was extracted using a 3% CTAB protocol modified from Yu and collaborators (Yu et al., 2012). The quantity and quality of the extracted RNA were determined using both fluorometer (Qubit 4, Thermo Fisher Scientific) and Nanodrop (Nanodrop 2000, Thermo Fisher Scientific) instruments. The cDNA synthesis was performed with an Affinity Script QPCR cDNA synthesis kit (Agilent Technologies) according the manufacturer’s instructions, beginning with 0.5 µg of RNA. The qPCR mix consisted of 2 µL of 10X buffer, 1 µL of MgCl$_2$ 50 mM, 0,75 µL of dNTPs 10 mM, 14,3 µL of nuclease-free water, 0,2 µL of Platinum Taq DNA Polymerase (Invitrogen) and 0,75 µL of primer mix. The primer sequences and qPCR conditions were obtained from the literature (Zamorano, 2013).
Results

In order to evaluate the antiviral properties against GRSPaV on *Vitis vinifera* plantlets cv. Cabernet Sauvignon, the effect of different concentrations of two Cu formats (CuSO$_4$ and NPs-Cu) on plant growth was studied. In addition, a damage scale was created to compare the stress caused by the different treatments. The foliar Cu content was measured in order to evaluate the metal mobility inside the plant. Finally, with these results, a test of efficiency of the designed protocol to eliminate GRSPaV from grapevine plantlets was performed.

*Evaluation of the effect of different Cu concentration on shoot development.*

To study the Cu effect on development of *in vitro* plantlets, increasing concentrations of CuSO$_4$ and NPs-Cu were supplemented to the basal medium. After the treatment, the following growth parameters were measured: Fresh plant weight, total shoot length, shoot number per plant and leaves number per shoot (Figure 1).

The results of fresh plant weight (Figure 1A) showed a significant reduction with 50 µM of Cu compared to the control plants. Higher Cu concentrations did not show an additional reduction of this parameter.

The total shoot length of plantlets treated with CuSO$_4$ (Figure 1C) exhibited a significant reduction in treatments since 15 µM of Cu compared to the control. Treatments with 75 µM of Cu showed a severe reduction of the total shoot length compared to treatments with lower Cu concentrations.
Regarding the shoot number per plantlet (Figure 1E) and the leaves number per shoot (Figure 1G), no significant differences were observed for the different CuSO₄ treatments.

The same growth parameters were measured in plantlets treated with different NPs-Cu concentration. As it is possible to appreciate in Figure 1, no differences were observed for the fresh weight, shoot length, shoot number per plantlet or leaves number per shoot (Figure 1B, D, F and H, respectively).

**Evaluation of the effect of different Cu concentration on the root development.**

In order to evaluate the Cu effect on root development, the following parameters were measured: Total root length, main roots number per plantlet and lateral root number per main root.

For CuSO₄ treatments (Figure 2A), a reduction of the main root length was observed in plantlets exposed to 15 µM of Cu compared to the control. For higher concentrations, the results showed that as the Cu concentration increased, the root length was significantly decreasing.

In the case of the main root number per plantlet (Figure 2C) a non-significative increase was observed in plantlets exposed to 5-50 µM of Cu in comparison to the control grapevines. In the other hand, treatments with 75 and 100 µM of Cu non-significantly reduced the main root number compared to the control. Additionally, it was possible to observe a significant difference between the treatments with 15 and 50 µM of Cu. Finally, a reduction of lateral root number was observed for all CuSO₄ treatments compared to the control.
When the development root parameters were measured in plantlets treated with NPs-Cu, no differences were observed in the main root length (Figure 2B), main root number per plantlet (Figure 2D) nor lateral root number per main root (Figure 2F). This result was consistent for all the different NPs-Cu concentrations evaluated.

**Evaluation of the damage caused by Cu treatments**

A damage scale was created to compare the plant stress level in response to the treatments. The 1 was assigned when an optimal growth and no stress signal were observed. The level 5 indicates that a plant dead caused by the metal exposition.

In the case of CuSO$_4$ treatments a significative damage was observed in plantlets treated with 75 and 100 µM of Cu (Figure 3A). However, some no significative alterations were observed from 15 µM of Cu. By contrast, plantlets exposed to the different concentrations of NPs-Cu did not show differences in the appearance and plant growth (Figure 3B). In additional experiments, all plants exposed to 100 µM of Cu died after a few days.

**Foliar Cu quantification**

To estimate indirectly the uptake and mobilization of Cu inside the plant a foliar Cu quantification was performed. The Cu concentration quantified in plantlets treated with CuSO$_4$ increased as the Cu concentration added to the medium increases (Table I). Values of up to 332 mg of Cu per kilogram of foliar tissue was detected in plantlets treated with 100 µM of CuSO$_4$. 
For NPs-Cu treatments, similar results were observed, because the higher Cu concentration in the medium, the higher Cu concentration detected in the leaves (Table I). However, the foliar Cu accumulation in these plantlets was lower than measured for CuSO₄ treatments, obtaining values close to half of the measured in plantlets treated with CuSO₄.

**GRSPaV elimination rate**

The concentrations of 50 and 65 µM of Cu for CuSO₄ treatments and 40 and 45 µM of NPs-Cu were selected for the sanitation test. The survival rate was 33.3% and 37.5% for the CuSO₄ treatments with 50 and 65 µM of Cu respectively. In addition, for 40 and 45 µM of NPs-Cu treatments the survival rates were 25% and 8.3% respectively (Table II). The viral detection analyses showed the presence of the GRSPaV in all the Cu treated plantlets.

**Discussion**

The results submitted here showed that different CuSO₄ concentration reduced the fresh plant weight and the total shoot elongation without modify the average leaves number per shoot or the lateral shoot number per plant. Also, a reduction of main root length and the lateral root number was observed. This decrease in plant growth is consistent with previous studies about the Cu effect on *V. vinifera* (Romeu-Moreno & Mas, 1999), *Prunus cerasifera* (Lombardi & Sebastiani, 2005) and *Withania somnifera* (Khatun et al. , 2008) plantlets, where also a reduction of chlorophyll content was observed.
In contrast, previous works had shown that NPs-Cu treatments reduced biomass and root elongation in crops such as *Cucurbita pepo* (Stampoulis, Sinha, & White, 2009), *Phaseolus radiatus* and *Triticum aestivum* (Lee *et al*., 2008). Similar results have been observed in *Landoltia punctate* (Shi *et al*., 2011) and in floral species as *Elsholtzia splendens* (Shi *et al*., 2013). However, in the present work the NPs-Cu treatments did not alter the shoot or root development nor did generate phenotypic damage on the plantlet leaves, although higher concentrations (100 µM of Cu) were lethal for the plant.

The Cu detected on leaves was closely related to the supplemented Cu concentration for both metal formats, which indicates that there is an adequate mobility of the compound towards the shoot, a necessary aspect for this metal to be used as a sanitation tool. However, the proportion of foliar Cu detected in CuSO₄ treated plantlets was higher than the quantified Cu on NPs-Cu treated plantlets. These results are contrary to the published by Shi *et al.* (2011), where a greater Cu accumulation was detected in duckweeds treated with NPs-Cu compared with the CuCl₂ treatments. However, the big morphologic and anatomic differences between *Landoltia punctata* (duckweeds) and *Vitis* genus make it difficult to establish a comparison of both results.

It have seen that plants exposed to high Cu concentrations accumulate the metal as precipitates in vacuoles and chloroplasts (Andrade *et al*., 2004) as a Cu immobilization mechanism to minimize its cell toxicity. In addition, the Cu antiviral potential require a direct contact with the pathogen. The results here observed indicate that both Cu formats are able to move to the foliar tissue, however, none of
them could eliminate the GRSPaV from *V. vinifera* cv. Cabernet Sauvignon. Considering the above, it is highly probable that the protocol performed here was not able to favor a direct contact between GRSPaV, a strictly phloemic virus, and the Cu accumulated in vacuoles and leaf chloroplasts.

Therefore, the methodology developed here did not allow efficient evaluation of the Cu viricidal potential against GRSPaV. For this reason, a simpler system and direct exposure of Cu with this or other plant viruses is recommended, to determine the Cu ability to eliminate grapevine viruses. Finally, at a later stage, establish a system that optimizes the contact of both parts within the plant material.

The results submitted in this work describe for the first time the effect of NPs-Cu treatments in a *in vitro* system. Although the results obtained here were not able to prove the viricidal potential of Cu against GRSPaV with the conditions evaluated here, several new uses could be evaluated with the protocol here developed.
Figure 1. Effect of copper treatments on shoot development of *in vitro* plants. Fresh weight (A and B), shoot length (C and D), number of shoots per plant (E and F) and number of leaves per shoot (G and H) were measured in plants treated with CuSO₄ (A, C, E and G) and NPs-Cu (B, D, F and H). ANOVA was performed and mean ± error was graphed. Different letters above bar indicate significant differences between treatments (p < 0.05).
Figure 2. Effect of copper treatments on root development of \textit{in vitro} plants. Root length (A and B), number of major roots per plant (C and D) and number of lateral roots per major root (E and F) were measured in plants treated with CuSO$_4$ (A, C and E) and NPs-Cu (B, D and F). ANOVA was performed and mean ± error was graphed. Different letters above bar indicate significant differences between treatments ($p < 0.05$).
Figure 3. Level of damage caused by copper treatments on plant development. A damage scale was designed with 5 levels, where 1 mean no damage on plant and 5 indicate plant death. The damage level was measured in plants treated with CuSO$_4$ (A) and NPs-Cu (B). ANOVA was performed and mean ± error was graphed. Different letters above bar indicate significant differences between treatments (p < 0.05).
Tables

Table I. Quantification of foliar copper in plants treated CuSO\(_4\) and NPs-Cu.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[Cu] in Leaves (mg/Kg)</th>
<th>Treatment</th>
<th>[Cu] in Leaves (mg/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>9</td>
<td>MS</td>
<td>5</td>
</tr>
<tr>
<td>MS + 5 uM CuSO(_4)</td>
<td>31</td>
<td>MS + 5 uM NPs Cu</td>
<td>15</td>
</tr>
<tr>
<td>MS + 15 uM CuSO(_4)</td>
<td>52</td>
<td>MS + 15 uM NPs Cu</td>
<td>25</td>
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<tr>
<td>MS + 50 uM CuSO(_4)</td>
<td>126</td>
<td>MS + 25 uM NPs Cu</td>
<td>32</td>
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<tr>
<td>MS + 75 uM CuSO(_4)</td>
<td>195</td>
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<tr>
<td>MS + 100 uM CuSO(_4)</td>
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<td>MS + 45 uM NPs Cu</td>
<td>48</td>
</tr>
<tr>
<td>--</td>
<td>--</td>
<td>MS + 55 uM NPs Cu</td>
<td>62</td>
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</tbody>
</table>
**Table II.** Survival rate and elimination of GRSPaV detected by qPCR from Cabernet Sauvignon grapevines treated with selected concentration of CuSO₄ and NPs-Cu.

<table>
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<th>Treatment</th>
<th>Survival Rate</th>
<th>GRSPaV elimination</th>
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<td>50 uM CuSO₄</td>
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<td>0/8</td>
</tr>
<tr>
<td>65 uM CuSO₄</td>
<td>9/24</td>
<td>0/9</td>
</tr>
<tr>
<td>40 uM NPs-Cu</td>
<td>6/24</td>
<td>0/6</td>
</tr>
<tr>
<td>45 uM NPs-Cu</td>
<td>2/24</td>
<td>0/2</td>
</tr>
</tbody>
</table>
References


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General conclusions

GRSPaV is one of the most prevalent grapevine viruses, mainly transmitted through contaminated plant material and highly recalcitrant to conventional sanitation techniques. However, the certification programs that regulate the sale of plant material have eliminated this virus from the list of pathogens that must be absent in the certificated plants. The main reason for this, is the lack of clarity about the real impact of the virus on V. vinifera, since although GRSPaV has been defined as the causal agent of different grapevine diseases, it has seen that a high percentage of the GRSPaV-infected plants develop asymptomatic infections. Furthermore, recent studies have proposed a co-evolution between both organisms, which would have allowed the establishment of a symbiotic mutualistic interaction between GRSPaV and V. vinifera. However, there are many doubts about this, and new information is required to confirm this hypothesis.

The present work sought to provide additional information about the effect of simple and mixed GRSPaV infections in early stages of plant development in one of the most important varieties for the country, the cultivar Cabernet Sauvignon. The results obtained here showed that simple infections of GRSPaV increased the grapevine growth, probably through an over-expression of the GA response pathway, a hormone related to the plant elongation. The presence of the virus did not affect the gaseous exchange parameters of the plant, probably because an over-expression of genes associated with the photosynthetic processes.
On the other hand, in plants with mixed infections no differences were observed in the growth nor in the expression levels of genes involved in the GA response pathway compared to healthy plants. However, mixed infections reduced the plant ability to fix the incorporated CO$_2$, even when no differences were observed in the expression levels of genes associated with photosynthesis (such as \textit{LHCII}) compared to healthy plants.

In conclusion, simple GRSPaV infections did not affect the photosynthetic rate of asymptomatic vines. Furthermore, our results indicate that these infections increased the growth in early stages of plant development. However, mixed infections of GRSPaV and GFLV reduced photosynthetic levels and exhibited similar growth rate compared to healthy plants.

Therefore, our results would be confirming a possible beneficial role of the virus in the plant vigor. However, when combined with GFLV, this effect is diluted, and the growth was similar to that of healthy plants. This could mean that the positive effects of asymptomatic GRSPaV infections compensate for some of the typical symptoms of GFLV infections, such as loss of vigor. But additional experiments are necessary to validate this hypothesis and confirm that this effect is maintained at over the years. On the other hand, the comparison of these results with plants infected only with GFLV are necessary to adequately describe the GFLV effects on the Cabernet Sauvignon plants and to be able to affirm a compensatory effect of the GRSPaV.

In the second part of our work, we sought to develop a new methodology for eliminate GRSPaV, using copper as an antiviral agent. The results obtained showed
that the cationic copper exhibited a clear effect both in the shoot and root development and high levels of mobility from the medium to the foliar tissue. By contrast, no differences in the plant growth were observed with the copper nanoparticles treatments, although this may be a consequence of the lower copper mobility through the plant.

Finally, none of the copper formats evaluated was able to eliminate the GRSPaV from the samples analyzed, probably because this protocol was not able to promote direct contact between the metal and the virus. However, it is not possible to rule out the viricidal potential of copper against GRSPaV, so an evaluation in a simpler system, such as a direct exposure of the virus to copper solutions, is suggested.