Universitat de les Illes Balears

DOCTORAL THESIS

2017

INTERACTIVE EFFECTS OF GRAPEVINE LEAFROLL ASSOCIATED VIRUS-3 (GLRaV-3) AND WATER STRESS ON THE GAS EXCHANGE, WATER USE EFFICIENCY, PLANT HYDRAULICS AND METABOLISM IN LOCAL GRAPEVINE CULTIVARS

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Ph. Doctor by the Universitat de les Illes Balears

الى امي، من اصدق قلبي أهديك الشكر و الامتنان تمجيدا لعطائك الكريم وحبك اللامتناهي

الى ابي،شكرالانك وضعتني على الطريق الصحيح قبل ان ترحل، تاركا ايانا امرأة عظيمة تسير بنا الى بر الامان

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SYMBOLS AND ABBREVIATIONS LIST

Symbols	Meaning
α	leaf absorptance
ABA	abscissic acid
Ala	alanine
A_N	net CO_2 assimilation
AOX	alternative oxidase
Arg	arginine
Asp	aspartic acid
ATP	adenosine triphosphate
B_L	biochemistry limitation
β	partitioning of absorbed quanta between photosystems II and I
C	calose
C_a	atmospheric CO_2 concentration
Caf	caffeic acid
C_c	chloroplast CO_2 concentration
CC	companion cells
Chl	chlorophyll
Chl a	chlorophyll a
Chl b	chlorophyll b
C_i	sub-stomatal CO_2 concentration
CO_2	carbon dioxide
COX	cytochrome oxidase
CP	coat protein
CPC	crystalline protein cluster
CPm	minor coat protein
Ct	threshold cycle
CTV	citrus tristeza virus
CW	cell wall
Δ_a	oxygen isotope fractionation of the alternative oxidase pathway
Δ_c	oxygen isotope fractionation of the cytochrome oxidase pathway
DIECA	na-diethyl-dithio-carbonate
D_L	density of the leaves
DTT	dithiothreitol
E	leaf transpiration rate
ELISA	enzime-linked inmunosorbent assay
ER	endoplasmic reticulum
Φ_{CO2}	apparent quantum efficiency of CO_2 fixation
Φ_{PSII}	photochemical efficiency of photosystem II
F_o	fluorescence signal when all reaction centers were open
f_{ias}	volume fraction of intercellular air spaces
F_M	fluorescence signal when all reactions centers were closed
$F_{M'}$	maximum fluorescence during a light-saturating pulse
F_s	steady-state fluorescence

Symbols	**Meaning**
F_V/F_M	maximum quantum efficiency of photosystem II
Γ^*	CO_2 compensation point in the absence of mitochondrial respiration
GC-TOF-MS	gas chromatography-time of flight-mass spectrometry
GFkV	grapevine fleck virus
GFLV	grapevine fanleaf virus
GLD	grapevine leafroll disease
GLRaVs	grapevine leafroll associated viruses
Glu	glutamic acid
Gly	glycine
GRSPaV	Grapevine rupestris stem pitting associated virus
g_s	stomatal conductance
gm	mesophyll conductance
GVA	grapevine virus A
GYSVd-1	grapevine yellow speckle viroid 1
His	histidine
hsp 70	heat shock protein 70
HRM	high-resolution melting
IC-PCR	immuno-capture PCR
Ile	isoleucine
JA	jasmonic acid
J_{max}	maximum capacity for electron transport rate
Kc	rubisco michaelis–menten constants for carboxylation
Ko	rubisco michaelis–menten constants for oxygenation
LAMP	loop-mediated amplification of nucleic acid
LEDs	light-emitting diodes
Leu	leucine
LMA	leaf mass area
L-Pro	leader protease
Lys	lysine
M	mitochondria
Mal	malate
MAP	mitogen-activated protein
MCFI	multicolour fluorescence imaging
MC_L	mesophyll limitation
MeOH	methanol
MEP	methylerythritol phosphate
Meth	methionine
mETC	mitochondrial electron transport chain
MRM	multiple reaction monitoring
MSTFA	n-methyl-n-(trimethylsilyl) trifluoroacetamide
MuLV	moloney murine leukemia virus
N	nucleus
NADH	nicotinamide adenine dinucleotide
O_2	oxygen
ORFs	open reading frames
PD	plasmodesmata
PEG	polyethylene glycol
Phen	phenylalanine
PPFD	photosynthetically active photon flux density

PPUs	pore plasmodesmal units
PR	pathogenesis-related
PSII	photosystem II
qPCR	quantitative PCR
R_d	leaf dark respiration
RdRp	RNA dependent RNA polymerase
ROS	reactive oxygen species
RT-PCR	reverse transcription-PCR
RuBP	ribulose biphosphate
RW	rugose wood
SA	salicylic acid
SDS	sequence detection systems
SE	sieve elements
SEL	size exclusion limit
Ser	serine
sgRNAs	small guides RNAs
S_L	stomatal limitation
+ss	positive sense single stranded
SSCP	single-strand conformation polymorphism
τ_a	electron partitioning through the alternative pathway
TA	titratable acidity
TBE	tris-borate-EDTA
Thre	threonine
T_L	total limitation
T_{mes}	mesophyll thickness
TSO_2	total sulphur dioxide
TSS	transcriptional start sites
Val	valine
V_{alt}	activity of the alternative oxidase pathway
$V_{c,max}$	maximum carboxylation capacity
V_{cyt}	activity of the cytochrome oxidase pathway
VP	vascular parenchyma cells
V_t	total oxygen uptake rate
V_{TPU}	rate of triose-phosphate utilization
w	width of leaf anatomical section
Ψ_{PD}	predawn leaf water potential

LIST OF PUBLICATIONS DERIVED FROM THE PRESENT THESIS

This thesis has been developed with a predoctoral fellowship (FPI-CAIB). Results obtained in the present thesis have resulted in the following papers:

1. **El Aou-ouad H**., Montero R., Medrano H., Bota J. (2016). Interactive effects of grapevine leafroll-associated virus 3 (GLRaV-3) and water stress on the physiology of *Vitis vinifera* L. cv. Malvasia de Banyalbufar and Giro-Ros. *Journal of Plant Physiology* **196**: 106- 115.

2. **El Aou-ouad H**., Pou A., Tomàs M., Montero R., Ribas-Carbó M., Medrano H. & Bota J. (2017). Combined effect of virus infection and water stress on water flow and water economy in grapevines. *Physiologia Plantarum.* doi:10.1111/ppl.12541

3. **El Aou-ouad H**., Montero M., Baraza E. & Bota J. (2016). Sanitary status of majorcan local grapevines cultivars and elimination of multiple infections from two *Vitis vinifera* cultivars combining thermotherapy with shoot tip culture. *Europrean Journal of Plant Pathology* (submitted)

4. **El Aou-ouad**., Florez-Sarasa I., Obata T., Montero R., Fernie A.R., Medrano H., Pou A. & Bota J. (2016). Physiological and metabolic changes in grapevines under combined drought stress and virus infection. *Frontier in Plant Science* (submitted)

CONTENTS

SUMMARY

Worldwide, agriculture production continues to be constrained by a number of abiotic and biotic stresses. In areas with Mediterranean climate, drought events are expected to strengthen in terms of intensity, frequency and duration in the near future. Thus, in this area, soil water deficit is considered the main environmental factor influencing grapevine growth. At the same time, grapevines are highly subjected to different viruses from which *Grapevine leafroll associated virus-3* (GLRaV-3) is one of the most widespread ones, provoking considerable economic losses in many vineyards worldwide. The identification of how different stress responses are integrated and how they affects plant growth and physiological traits, is therefore very important to ensure the continuity of vine growing. The first main objective of the present thesis was to study the sanitary status of Majorcan minority grapevines cultivars and to highlight the prevalence of GLRaV-3 in local cultivars. In this sense, the results showed that virus incidence is very high and can rise up (91.75 %) in the local grapevine cultivars conserved in the germplasm collection of the Balearic Islands. Remarkably, GLRaV-3 is the most prevalent virus in this collection (82 %). This situation urges a speedy implementation of the sanitation techniques in order to obtain virus-free certified plants. Hence, two sanitation techniques "shoot tips culture and thermotherapy in combination with shoot tips culture" have been optimized for double and triple viruses' eradication from two local cultivars with high enological potential, Argamussa and Gorgollassa.

The second main objective was to dissect the effects of virus infection, moderate water stress and its combination on main physiological processes in two local grapevine cultivars, Malvasia de Banyalbufar and Giró Ros. Each of the two studied stresses resulted in a negative effect on leaf gas-exchange parameters. Water stress in non-infected plants (WS-NI) significantly reduced plant growth and net CO_2 assimilation (A_N) but had only small effect on metabolic changes, indicating that A_N changes were mainly constrained by diffusive parameters (stomatal (g_s) and mesophyll (g_m) conductance's). With regard to virus infection under well-watered conditions (WW-VI), the results also revealed that GLRaV-3 impaired A_N through diffusional limitations rather than biochemical ones. In this case, stomatal conductance was mainly regulated by changes in hydraulic conductance at leaf level, namely leaf (K_{leaf}) and petiole ($K_{petiole}$) hydraulic conductance, in which $K_{petiole}$ resulted to be the most relevant parameter to be checked when studding the effect of phloemetic viruses in plant

hydraulics. Remarkably, the results of this thesis indicate that the combined stress resulted in a detrimental effect on plant growth and physiology; but any additive effect between WS and VI has been highlighted. Moreover, the combination of stresses triggers a specific response at the metabolic level, which was not quantitatively predicted by the sum of each individual stress. The observed changes in primary metabolites were closely associated with respiration metabolisms. Indeed, the specific adjustment of respiratory metabolism (i.e; cysteine, threonine, fumarate and erythronate 1,4-lactone) could potentially explain the maintenance of leaf carbon balance and growth in cultivars studies under combined stress conditions. In the present thesis, we move a step further, by revealing the importance of studding grapevine-virus infection-water stress interactions under field conditions, particularly, in white cultivars where viruses are asymptomatic and difficult to identify. Further research with differents virus concentration and high number of cultivars are needed to confirm if virus effect is concentration dependent and if there is a cultivar-susceptibility difference.

RESUMEN

A escala mundial, la producción agrícola sigue estando limitada por una serie de estreses abióticos y bióticos. En las zonas de clima mediterráneo, es muy probable que, en un futuro próximo, la duración, la frecuencia y/o la intensidad de la sequía aumenten. De esta forma, en el mediterráneo, el déficit hídrico se considera el principal factor ambiental limitante del crecimiento de la vid. Al mismo tiempo, el cultivo de la vid está expuesto a diferentes enfermedades provocadas por virus, de los cuales el virus del enrollado 3 (GLRaV-3) es uno de los más extendidos, provocando importantes pérdidas económicas en muchos viñedos de grandes regiones vitícolas de todo el mundo. La identificación de cómo se integran las respuestas de diferentes estreses en la viña y también de cómo están afectando el crecimiento y la fisiología de las plantas es, por tanto, de gran interés para garantizar la continuidad del cultivo de la vid. El primer objetivo de esta tesis es estudiar el estado sanitario de las variedades minoritarios de la vid y destacar la incidencia del GLRaV-3 en las variedades locales de vid. Los resultados obtenidos han puesto de manifiesto que la incidencia de los virus es muy alta (91,75%) en las variedades locales de vid conservadas en la colección de germoplasma de las Islas Baleares. Sorprendentemente, en esta colección, GLRaV-3 es el virus que mostró una mayor incidencia (82%). En este sentido, instamos a la rápida aplicación de técnicas adecuadas de saneamiento para obtener plantas certificadas libres de virus. Por ello, se han optimizado dos técnicas de saneamiento " cultivo *in vitro* de ápices caulinares y la combinación de termoterapia y cultivo *in vitro* de ápices caulinares" para la erradicación de las infecciones víricas dobles y triples de dos cultivares locales de gran potencial enológico, Argamussa y Gorgollassa.

El segundo objetivo principal de esta tesis es explorar los efectos del estrés hídrico, la infección vírica y la combinación de ambos sobre los principales procesos fisiológicos de dos variedades locales de vid, Malvasia de Banyalbufar y Giró Ros. Cada uno de los dos estreses estudiados resultó en un efecto negativo sobre los parámetros de intercambio gaseoso foliar. El estrés hídrico in plantas sanas (WS-NI) redujo significativamente el crecimiento de las plantas y la asimilación neta de CO_2 (A_N), pero solo demostró tener un efecto reducido sobre los niveles metabólicos, indicando en este caso, que los cambios de A_N durante el WS se ven limitados por factores difusivos (la conductancia estomática (g_s) y del mesofilo (g_m)). Respecto a las plants infectadas en condiciones de riego (WW-VI) las correlaciones obtenidas entre los parámetros

fisiológicos (A_N, g_s y g_m), la concentración de virus y los metabolitos sugiere firmemente que el GLRaV-3 afecta a la A_N mediante limitaciones difusivas más que bioquímicas. En este caso, la conductancia estomática fue regulada principalmente por la conductancia hidráulica a nivel foliar, concretamente conductancia hidráulica de hoja (K_{leaf}) y pecíolo ($K_{petiole}$). En este trabajo, $K_{petiole}$ resultó ser el parámetro más relevante para estudiar el efecto de los virus sistémicos de tipo floemático sobre la hidráulica de la planta. Notablemente, los resultados de esta tesis indican que el estrés combinado tuvo un efecto negativo sobre el crecimiento y la fisiología de la vid; pero no se destacó ninguna interacción entre WS y VI. Por el contrario, la combinación de los dos estreses subrayó una respuesta específica a nivel metabólico, no pudiéndose predecir cuantitativamente este efecto en base a la suma de cada uno de ellos. Los cambios observados en los metabolitos primarios se asociaron estrechamente con el metabolismo respiratorio. De hecho, el ajuste específico del metabolismo respiratorio (-i.e, cisteína, treonina, fumarato y eritronato 1,4-lactona) podría explicar potencialmente el mantenimiento del balance de carbono foliar y el crecimiento de las variedades estudias bajo condiciones de estrés combinado. En la presente tesis, hemos revelado la importancia de estudiar las interacciones vid-virus-estrés hídrico en condiciones de campo, particularmente en las variedades blancas donde las infecciones son asintomáticas y difíciles de identificar. Para completar este trabajo, sería interesante realizar un experimento con diferentes concentraciones de virus en un mayor número de cultivares y así confirmar si el efecto del virus depende de la concentración y/o si hay una diferencia en la susceptibilidad entre los cultivares.

RESUM

Actualment, la producció agrícola mundial es troba limitada per una sèrie d'estressos abiòtics i biòtics. A les zones de clima Mediterrani, s'espera que en un futur pròxim els esdeveniments de sequera siguin encara de major importància en termes d'intensitat, freqüència i durada. Per tant, en aquestes zones, el dèficit hídric és considerat com el principal factor ambiental capaç d'afectar el creixement de la vinya. A més, la vinya es troba altament sotmesa a diferents virus, entre els quals, *Grapevine leafroll-associated virus-3* (GLRaV-3) és un dels més estesos, provocant pèrdues econòmiques considerables a moltes vinyes d'arreu del món. La identificació de com les diferents respostes a l'estrès es troben integrades a la vinya i de com aquestes afecten el creixement i la fisiologia de les plantes és, per tant, de gran interès per a garantir la continuïtat del cultiu de la vinya. El primer objectiu d'aquesta tesi tracta d'estudiar l'estat sanitari dels cultivars minoritaris de vinya a Mallorca i destacar la prevalença a la vinya del virus GLRaV-3. Aquest estudi demostra una incidència d'infeccions víriques simples i múltiples (91,75%) molt alta en els cultivars locals de vinya, actualment conservats a la col·lecció de germoplasma de les Illes Balears. Sorprenentment, GLRaV-3 és el virus que més predomina en aquesta col·lecció (82%). Aquest resultat impulsa la necessitat d'una aplicació dràstica de tècniques de sanejament per tal d'obtenir plantes certificades lliures de virus. En aquest treball, s'han optimitzat dues tècniques de sanejament "shoot tips culture and thermotherapy in combination with shoot tips culture" per a l'eradicació de virus dobles i triples a dos cultivars locals amb alt potencial enològic, Argamussa i Gorgollassa.

El segon objectiu principal d'aquesta tesi és estudiar els efectes de la infecció vírica, l'estrès hídric moderat i la seva combinació sobre els principals paràmetres fisiològics a dues varietats locals de vinya, Malvasia de Banyalbufar i Giró Ros. Cada un dels estressos estudiats, va resultar en un efecte negatiu sobre l'intercanvi de gasos a nivell foliar. L'estrès hídric a plantes sanes (WS-NI) va reduir de manera significativa el creixement de les plantes i l'assimilació neta al CO_2 (A_N), en canvi, sols va afectar lleugerament els canvis metabòlics, el que indicà que canvis sobre la A_N durant l'estrès hídric foren principalment deguts a les limitacions sofertes sobre determinats paràmetres difusius, com son la conductància estomàtica (g_s) i la conductància del mesòfil (g_m). A les plantes infectades per virus i cultivades sota condicions de reg (WW-VI), s'obtingueren correlacions significatives entre els paràmetres fisiològics (A_N,

g_s i g_m), la concentració de virus i la presència de metabòlits. Aquestes correlacions varen desvelar que la presència de GLRaV-3 afectà la A_N a través de limitacions difusives i no bioquímiques. En aquest cas, la conductància estomàtica es regí principalment pels canvis en la conductància hidràulica a nivell de la fulla (K_{leaf}) i el pecíol ($K_{petiole}$), essent $K_{petiole}$ el paràmetre més rellevant alhora d'estudiar l'efecte de virus floemàtics sobre el sistema hidràulic de la planta. Cal remarcar que els resultats obtinguts en aquest treball indiquen que la presència d'un estrès combinat tingué un efecte negatiu sobre el creixement i la fisiologia de la vinya; però en cap cas es destacà una interacció entre WS i VI. Per contra, la combinació d'ambdós estressos desencadenà una resposta específica a nivell metabòlic, que no va ser quantitativament predita per la suma dels dos. Els canvis observats en els metabòlits primaris varen resultar estar estretament associats amb el metabolisme respiratori. De fet, l'ajust específic del metabolisme respiratori (és a dir, cisteïna, treonina, fumarat i eritronat 1,4-lactona) podria explicar el manteniment de l'equilibri entre el balanç de carboni i el creixement en els dos cultivars estudiats. En el present treball, s'ha avançat en quant a revelar quant d'important és l'estudi de les interaccions virus-estrès hídric a la vinya en condicions de camp, en particular, en els cultivars de raïm blancs on els efectes del virus són asimptomàtics i més difícils d'identificar. En un futur, es necessitaria aprofundir en aquest camp mitjançant la realització d'estudis addicionals amb diferents concentracions víriques i un major nombre de cultivars per a ser capaços de confirmar si l'efecte del virus sobre la vinya depèn de la seva concentració i si cada cultivar presenta diferent susceptibilitat a la presència del virus.

INTRODUCTION

1. CURRENT STATUS OF GRAPEVINE LOCAL CULTIVARS, RECUPERATION AND CONSERVATION

The European grapevine (*Vitis vinifera* L.) is considered one of the oldest and most important crops worldwide. As reported by the International Organization of Vine and Wine (OIV, 2014), the total harvested area of grapes in the world was estimated to be around 7.5 million hectares. Moreover, *V. vinifera* L. is considered the most dominant species among all the different species of grapevine cultivated, which is planted for making wine (70%), producing fresh market table grapes (22%) and raisins (8%) (Troggio et al. 2008). At the end of 19th century, different diseases agents from America reached Europe (Powdery mildews, *Phylloxera* (*Phylloxera vastatrix*)) and lead to a huge devastation and destruction of many European vineyards, inducing a drastic change in the diversity of cultivated and wild grapes. However, presently, it has been reported by Smart (2013) that the extent of the problem of trunk diseases is much stronger than phylloxera in different vineyard regions of Australia. Others factors had also led to a substantial decrease in grapevine diversity, producing an important genetic erosion of the gene pool (This et al. 2006). Indeed, a second wave of genetic diversity loss was occurred over the last 50 years, owing to the globalization of wine and quality demarcation of a number of cultivars and vineyard areas. The emergence of the few cultivars grown worldwide such as Chardonnay, Cabernet Sauvignon, Syrah and Merlot are increasing and inducing at the same time the disappearance of old local cultivars (Cipriani et al. 2010; Terral et al. 2010; García-Munõz 2011). On the other hand, sanitary selection of healthy disease-free clones has also induced a reduction in clonal diversity for these major cultivars around the world. Furthermore, it is shown that the use of few cultivars admitted by the different Designations of Origin (D.O) have also contributed to a substantial decrease in grapevine diversity and marginalization of local cultivars in many growing area, including Spain (Prota et al. 2010; Moreno-Sanz et al. 2011); even if they are perfectly adapted to the local environmental condition and playing an important role in the diversification of wines (Cabello 2004).

In the Balearic Islands, the viticulture area was reduced from 30.000 ha in the 19th century to 2000 ha with the *Phylloxera* attack. In the 60s, Majorcan grapevine area has been received another blow. Most vineyards were abandoned owing to the boom of tourism and the investment in hotel industry and construction. It is mentioned above

that the homogenization of international wine market also contributed to the accentuated erosion of local grapevine cultivars in the Balearic Islands (García-Muñoz et al. 2012). However, despite all these alterations and the reduced geographic area, the grapevine diversity found in the Balearic Island is considered very high (García-Muñoz 2011).
Since Roman times, the Balearic wines are well known in the world for their high quality (Hidalgo 2002). Several studies have been shown the high oenological aptitude of some minor cultivars (Escalona et al. 2009, 2012; Bota et al. 2013). In 2014, García-Muñoz et al. reported also the high wine quality of 18 minor varieties of the Balearic Islands. In addition, nowadays the wine consumers are looking for a new product based on the originality, quality link to the '*terroir'* and historical background, thus the use of local varieties could be a paramount factors to fill this gap and to satisfy D.O requirement, being also one of the last opportunities to preserve them in the future (Santiago et al. 2008).

Interestingly, the knowledge of the existing genetic diversity in vineyards is considered a priority when addressing its conservation and revalorization. In order to overcome this situation, germplasm banks have played an important role in the conservation of grapevine diversity (This et al. 2006; Maghradze et al. 2010). Several studies on the surveying, recuperation, characterization and maintaining of cultivars in germplasm banks are being carried out worldwide (Aradhya et al. 2003; Halász et al. 2005; Heuertz et al. 2008; Leão et al. 2009; Maletic et al. 1999; Zdunić et al. 2008; Alifragkis et al. 2015; Brunori et al. 2015); including the conservation of major and minor cultivars in different Spain regions (Buhner-Zaharieva et al. 2010; Maghradze et al. 2010; Cretazzo et al. 2010c; Prota et al. 2010; Casanova et al. 2011; García-Muñoz et al. 2012; Loureiro et al. 2011; Sivcev et al. 2011; Moreno-Sanz et al. 2011; Bota et al. 2013; Balda et al. 2014; Urrestarazu et al. 2015).

1.1. Abiotic stress: Incidence of water limitation

Abiotic stress is defined as the non-living environmental conditions responsible for the growth and yield reduction below optimum levels. Under field conditions, the main abiotic stresses affecting plants and crops are being extensively studied (Cavanagh et al. 2008; Munns and Tester 2008; Chinnusamy and Zhu 2009; Mittler and Blumwald 2010). This includes water stress, salinity, heat, cold, chilling, freezing, nutrient, high light intensity, ozone (O_3) and anaerobic stresses (Wang et al. 2003; Chaves and

Oliveira 2004; Agarwal and Grover 2006; Nakashima and Yamaguchi-Shinozaki 2006; Hirel et al. 2007; Cramer et al. 2011; Carvalho et al. 2015). A report by the Food and Agricultural Organization in 2007 stated that only 3.5% of the global land area is free from any environmental constraints (http://www.fao.org/docrep/010/a1075e/a1075e00.htm). Considering the percentage of land area affected and loss of crop productivity, study of abiotic stresses and its management continue to be a significant area of research in plant biotechnology (Table 1).

Table 1 Estimates of the impacts of abiotic stresses on crop production and published research

Stress Type	% of global land area affected*	% of global rural land area affected**	Number of Publications***
Abiotic Stress		96.5	**35,363**
Water			**4819**
Deficit or Drought	64	16	4137
Flooding or Anoxia	13	10	682
Temperature			**9715**
Cold	57	26	3798
Chilling			187
Freezing			350
High or heat			5380
Light			**7659**
Low			3081
High			4578
Chemical/Soil		50	**12391**
Salt or salinity	6	6	3498
Mineral deficiency or low fertility	9	39	222
Mineral toxicity			437
Acid soil	15		3646
Air pollutants			
Ozone			1369
Sulfur dioxide			378
NO_x oxide			2001
Elevated CO_2			840
Miscellaneous (e.g. wind, mechanical, etc.)			**779**

*based on FAO World Soil Resources Report 2000 ftp://ftp.fao.org/agl/agll/docs/wsr.pdf.

** based on Tables three point six and three point seven of 2007 FAO Report http://www.fao.org/docrep/010/a1075e/a1075e00.htm

*** data based on simple searches in PubMed between 2001 and July 7, 2011.

Water limitation is one of the major threats in agricultural production, and this is projected to get considerably worse due to Climatic Change in coming decades (IPCC 2013). In Mediterranean climate areas, grapevines usually deal with water deficit during growth period because most of its growth season copes with summer (Chaves et al. 2007, Flexas et al. 2010). According to some predictions (Schultz 2000), the increase in temperature induced by the two-fold CO_2 concentration, would cause decreases in soil moisture content, from 20-30% for most of the Mediterranean areas, and up to 70% in

the Iberian Peninsula and Balearic Islands. This will lead to an over-exploitation of water resources for viticulture use, forcing the use of specific water-resistant cultivars and making irrigation necessary in areas where it is currently not available. In order to mitigate the negative impact of those changes on grape growth and quality, adaptations in wine-growing practices are needed. Local grapevine recuperation could be a good candidate to cope with water stress limitation as those old local cultivars are adapted to the traditional rainfed viticulture. Different physiological responses of local cultivars to water stress have been studied in many Spanish (Medrano et al. 2003; Gomes-del-Campo et. 2004; Islam and Berrios 2012; Martinez et al. 2016) and Portuguese regions (Chaves et al. 2003; Costa et al. 2012; Fraga et al. 2016).

In the Balearic Islands, a large effort has been made to explore the existing genetic variability in terms of water use efficiency (WUE), either in potted or field plants (Bota et al. 2001; Escalona et al. 1999; Tomás et al. 2012, 2014; Bota et al. 2016). Those works revealed the different potential resistances to drought of local cultivars as well as different capacities for better water use. Moreover, it has been pointed out that some ancient local cultivars can be good candidates for the current and future viticulture in semiarid conditions.

1.2. Biotic stress: Incidence of virus infection

In addition to abiotic pressures, plants have to face the threat of infection within their natural habitat and must defend themselves from the attack of different pathogens, including fungi, bacteria, viruses and herbivore pests (Hammond-Kosack and Jones 2000; Atkinson & Urwin 2012). Within the context of climate change, the habitat range of pests and pathogens can also be influenced by increasing temperatures, thus facilitating pathogen spread (Bale et al. 2002; Luck et al. 2011, Madgwick et al. 2011, Nicol et al. 2011; Smart 2013) and consequently producing important damages in plants. In 2004, Oerke and Dehne revealed that those pathogens (Bacteria, fungal and viruses) and animal pests' causes reductions of 15% and 18% of the crop yield (wheat, rice, maize, barley, potatoes, soybeans, sugar beet and cotton), respectively, resulting in vast impact in the global food production.

Grapevines are susceptible to a wide range of pathogens that cause diseases in pre-and post- harvest periods, affecting production, processing and export, along with fruit quality. Some of the most important diseases in *V. vinifera* are the gray mold,

powdery mildew, downy mildew (DM), caused by *Botrytiscinerea*, *Erysiphenecator* and *Plasmoparaviticola*, respectively, and viruses. To date, nearly 70 virus species have been identified and that are able to infect the *Vitis* genus, accounting for at least 25 different diseases in grapevine (Martelli 2014). From an economic point of view, the most important grapevine viruses are those who cause the leafroll diseases (GLD), known as Grapevine leafroll associated viruses (GLRaV -1, -2, -3, -4, and -7) (Naidu et al. 2015). This thesis has addressed special attention to the GLRaV-3 virus.

GLD is one of the most diseases affecting the productive life of grapevine plants, wine, juice, and table grape cultivars, as well as rootstocks (Andret-Link et al. 2004; Padilla et al. 2007; Cretazzo et al. 2010a; Naidu et al. 2014; Montero et al. 2016a). Indeed, the EU Directive 2002/11/EC rules require that the initial plant material for vegetative propagation it is virus-free, namely to Grapevine fanleaf virus (GFLV), Arabis mosaic virus (ArMV), Grapevine fleck virus (GFkV), Grapevine leafroll associated virus-1 (GLRaV-1), and Grapevine leafroll associated virus- 3 (GLRaV-3) (Peiró et al. 2015).

The sanitary status of many local cultivars has remained neglected and unexplored to date, leading to the deterioration and loss of certain cultivars (Komínek and Holleinová 2003; Cretazzo 2010; Bota et al. 2013; Salami et al. 2009; Mahfoudhi et al. 2014). It has been shown that the local grapevine varieties usually present large levels of virus infections (Poljuha et al. 2004; Materazzi et al. 2006; Zdunic et al. 2007; Laimer et al. 2009; Bertolini et al. 2010; Cretazzo 2010). In Majorcan viticulture, it's has been shown that the incidence of multiple and single viral infections was very frequent and that GLRaV-3 was the predominant virus in most local varieties (Cretazzo et al. 2010b), because of its higher replication efficiency compared to other grapevine leaf-roll viruses (Velasco et al. 2014).

2. WATER STRESS EFFECTS ON GRAPEVINE

Grapevine responses to water stress are complex, involving adaptive changes and/or deleterious effects. This complexity comes from the combined effect of water stress, high air temperature and high evaporative demand during summer in Mediterranean area, thus affecting grapevine yield, berry and wine quality (Escalona et al. 1999; Chaves et al. 2010; Flexas et al. 2010; Lovisolo et al. 2010). Grapevine responses to water stress have been widely studied at physiological and molecular levels in the last decades (Lawlor and Tezara 2009; Vandeleur et al. 2009; Lovisolo et al. 2010; Chaves et al. 2010). These responses can take place at two different levels (leaf and whole plant): (i) instantaneous control of transpirational flux via the stomata; and (ii) the ability to survive drought periods of several weeks, which depends on the long-term water relations between whole plant and the soil (Schulze et al. 1987).

Among the physiological drought avoidance mechanisms, stomatal control is identified as the most important adaptation (Medrano et al. 2003; Shultz, 2003a; Pou et al. 2012; Tomàs et al. 2012, 2014). Moreover, regulation of plant hydraulic conductivity (Lovisolo et al. 2002, 2008; Pou et al. 2012, 2013) and the active regulation of the osmotic pressure (Rodrigues et al. 1993; Patakas and Noitsakis 1999, 2001) have also been shown to contribute to the maintenance of open stomata and the improvement of grapevine performance under water stress conditions. The mechanisms involved in the response of grapevine to water stress depend on several factors such as, genotypes and drought intensity and timing. Under moderate stress, it has been shown that stomatal closure is restricting plant water loss and carbon assimilation (Chaves et al. 2003). It is fairly well-established that under mild to moderate water stress, photosynthesis reductions are mainly due to diffusive limitations -i.e. decreased stomatal and mesophyll conductance- (Flexas and Medrano 2002a; Chaves et al. 2002; Galmés et al. 2007d; Flexas et al. 2012). However, when the stress is intensified (severe water stress), photosynthesis limitations were mainly due to metabolic impairments (maximum velocity of Rubisco carboxylation - V_{cmax} and maximum electron transport rate - J_{max}, Bota et al. 2004). As a consequence, dramatic reductions in carbon assimilation, as well as partial losses of the total leaf canopy area are observed (Flexas et al. 1998, 2002; Chaves et al. 2007; Souza et al. 2003, 2005b; Santos et al. 2007).

Under water stress or very large evaporative demand conditions, the plant must adjust its water consumption according the present environmental conditions, to avoid large variations of water potential that can cause definitive damage to the xylem vessels (cavitation, Lovisolo and Schubert 1998; Cochard 2002). The stomatal control is partially performed via hormones produced under drought such as abscisic acid (ABA) (Davies and Zhang 1991; Tardieu and Simonneau 1998; Dodd 2005), but is also influenced by the leaf water potential (Buckley 2005; Brodribb and Cochard 2009; Rodriguez-Dominguez et al. 2016). The leaf capacity to conduct water is also influenced by the leaf hydraulic conductance (K_{leaf}). In recent years, most of the works on grapevines under limited water conditions have been focused on the relation between g_s, ABA and K_{leaf} (Correia et al. 1995; Lovisolo et al. 2008; Pou et al. 2008; Romero et al. 2012; Speirs et al. 2013; Tramontini et al. 2014). Indeed, ABA and hydraulic conductance have shown to be a paramount role on g_s regulation and therefore, leaf water use efficiency (WUE) in two cultivars Tempranillo and Grenache showing contrasting behavior (Martorell et al. 2015).

In parallel to physiological mechanism responding to water stress, technological advances in diverse metabolite profiling approaches, whether in grapevine or other crops plants, have been studied deeply to understand the plant-environment response at the molecular level, metabolic, phenotypic diversity and its underlying genetic variation (Obata and Fernie, 2012; Tohge et al. 2013b; Brunetti et al. 2013; Hochberg et al. 2015). Those studies have revealed an important role of plant metabolic regulation including regulation of photosynthesis and accumulation of many amino acids such as proline, raffinose family oligosaccharides and tricarboxylic acid (TCA) cycle metabolites in responce to drought stress. Drought elicites changes in plant metabolism were mostly studies in Arabidobsis, wheat, barley, tomato and Maiz. Nevertheless, only few studies have investigated the genotypic variability in the metabolic response to water stress in grapevine (Cramer et al. 2007; Chaves et al. 2009; Hochberg et al. 2013). For instance, recently, the metabolic response of grapevine to progressive water stress has been explored in two cultivars, Shiraz and Cabernet Sauvignon, which were shown to have different hydraulic behaviors (Hochberg et al. 2013).

3. GRAPEVINE LEAFROLL ASSOCIATED VIRUS 3 (GLRaV-3) EFFECTS ON GRAPEVINE

3.1. Grapevine leafroll associated virus-3

General approach

Grapevine leafroll disease (GLD) is one of the most serious viral diseases of grapevine, occurring in all grapevine-growing areas worldwide (Martelli 2000). There are evidences that GLD occurred in Europe and in other regions of the Mediterranean basin before the introduction of phylloxera (*Dactulosphaira vitifoliae*) in the mid nineteenth century (Gale 2002).

GLD is a complex viral disease producing different symptoms in red- and white-berried cultivars (Naidu et al. 2008). The severity of the symptoms can vary greatly depending on several factors like the season, cultivar and climatic conditions. In many red-berried cultivars, symptomatic leaves exhibit red or reddish-purple discolorations in interveinal areas, but primary and secondary veins remain green (Figure. 1A). The red and reddish-purple coloration of symptomatic leaves is due to the accumulation of specific classes of anthocyanin pigments. In contrast, white-berried cultivars show mild yellowing or chlorotic mottling of interveinal areas of leaves (Figure. 1B). These symptoms, however, are often subtle and may not be recognized in many white-berried cultivars, like in Thompson Seedless, Sauvignon Blanc, as well as Malvasía de Banyalbufar and Giró Ros; the two autochtonuous cultivars from the Balearic Islands and used in the current thesis. However, some cultivars like Chardonnay may show general yellowing or chlorotic mottling towards the end of the season. In both red- and white-berried cultivars, symptoms often appear first on mature leaves at the bottom portion of the canopy around *véraison* and progressively move upward to younger leaves as the season advances.

To date, a number of different viruses in the family *Closteroviridae* have been reported to be associated with GLD. These viruses include Grapevine leafroll associated viruses (GLRaV) 1–9 and a group of more recently described viruses (GLRaV-Pr, GLRaV-De, and GLRaV-Car). All these viruses belong to the genus *Ampelovirus* except for GLRaV-2 (genus *Closterovirus*) and GLRaV-7 (genus *Velarivirus*) (Al Rwahnih et al. 2011). Among the currently known GLRaVs, GLRaV-3 has been reported in almost all grapevine-growing regions worldwide, emerging as an

economically important constraint to the wine, table raisin, and nursery industries (Maree et al. 2013). Crop losses have been reported in several studies, between 14 and 40%, due to GLD infection (Wolpert et al. 1992; Martelli et al. 2012; Naidu et al. 2014). A recent economic study indicated that GLD, depending on the level of disease incidence, yield reduction, and impact on fruit quality, can cause an estimated loss of approximately $25,000 to $40,000 per hectare in the absence of any control measure (Atallah et al. 2012)

A  B 

Figure. 1: Leafroll disease symptoms in red cultivar *Vitis vinifera* Cabernet Franc cv. (A) and white cultivar *Vitis vinifera* Chardonay cv. (B) (From Maree et al. 2013)

Morphology and virion properties

The particles of GLRaV-3 are flexuous filaments with a non-enveloped virion of approximately 1,800x12nm in size, showing distinct cross banding (Figure. 2). The structure of filaments are helically constructed with a pitch of the primary helix of about 3.5 nm, containing approximately 10 protein subunits per turn of the helix (Martelli et al. 2011). The fragility of the virions and the tendency to end-to-end aggregation contributes to the fact that a range of lengths is often given by a single virus. This virus has been introduced to most grape growing regions by exchange and propagation of infected plant material and subsequent local spread by vegetative propagation and insect vectors (Cabaleiro and Segura, 2006; Martelli and Boudon-Padieu, 2006; Sharmaet al. 2011; Tsai et al. 2012).

Transmission

Overall, virus transmission is governed by several factors like virus accumulation, the propagation method (seeds or pollen, grafting, mechanical wounds and vectors), infection time and virus-host compatibility.

Mealybugs (insects in the family Pseudococcidae) were first shown to transmit Ampelovirus spp. in 1990 (Tsai et al. 2010). Since then, some mealybug and soft-scale (Coccidae) species have been shown to transmit different GLRaVs (Cabaleiro et al. 1994; Petersen and Charles, 1997). Transmission of GLRaV-3 has been demonstrated for various species of mealybugs (Pseudococcidae) and few species of soft scale insects (Coccidae) (Tsai et al. 2010).

However, GLRaV-3 can also be transmitted by graft and mainly spread by propagation of infected material. Direct damage to grapevines due to these mealybug species is primarily associated with infestation of the fruit clusters and growth of sooty molds as a consequence of honeydew excretion. Populations of these species are often kept low due to insecticide applications and natural enemies.

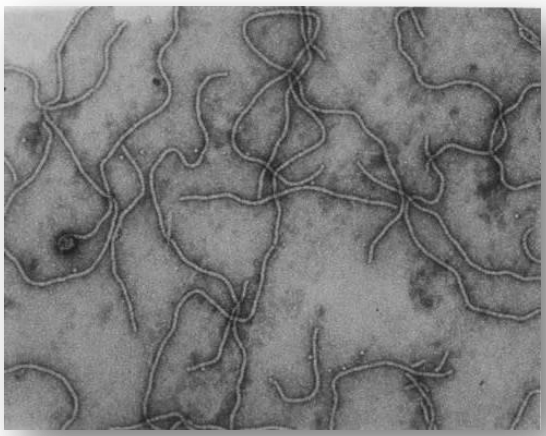

Figure. 2: Negatively stained of purified GLRaV-3 particles (From Maree et al. 2013).

Systemic transport of GLRaV-3

Systemic transport through the vascular system is a crucial step in plant virus infection. Several plant viruses, including GLRaV-3, take advantage of the transport of photoassimilates to move systemically through the phloem (reviewed by Haywood et al. 2002; Lucas and Wolf 1999; Nelson and Van Bel 1998; Oparka and Turgeon 1999; Thompson and Schulz 1999). Systemic transport implies firstly the entry into the source tissues of the phloem, then its circulation in the phloem transport and finally the exit

from the phloem to sink tissues. Consequently, the infection of different cell types occurs very easily (Ueki and Citovsky 2007 and Pallas et al. 2011). According to Maree et al. (2013), GLRaV-3 is restricted to the phloem of infected hosts (*V.vinifera* and American rootstocks), whose organs and tissues are unevenly distributed (Rowhani et al. 1997). The spread of this virus was also shown to be through both internal and external types of phloem. Indeed, the virus may spread either upwards to young sink tissue or downwards to the roots, with the former translocation being faster than the latter (Cheng et al. 2000). Virus transport in the phloem tissues, including GLRaV-3, takes place in two steps, locally, via cell-to-cell and through long distance movement. Virus entry into epidermal and mesophyll cells is followed by genome translation and replication. After that, virus move from cell to cell until reaching the sieve elements (SE) where they rapidly move to distant sites in order to establish the newly infection cells to finally infect the whole plant. Long distance movements, implies the crossing of viruses through several cellular barriers: the bundle sheath (BS), vascular parenchyma cells (VP), and companion cells (CC) (Figure. 3). To carry out cell-to-cell and long-distance movements, viruses take also advantage of plasmodesmata (PD) and follow the source-to-sink transportation of carbohydrates.

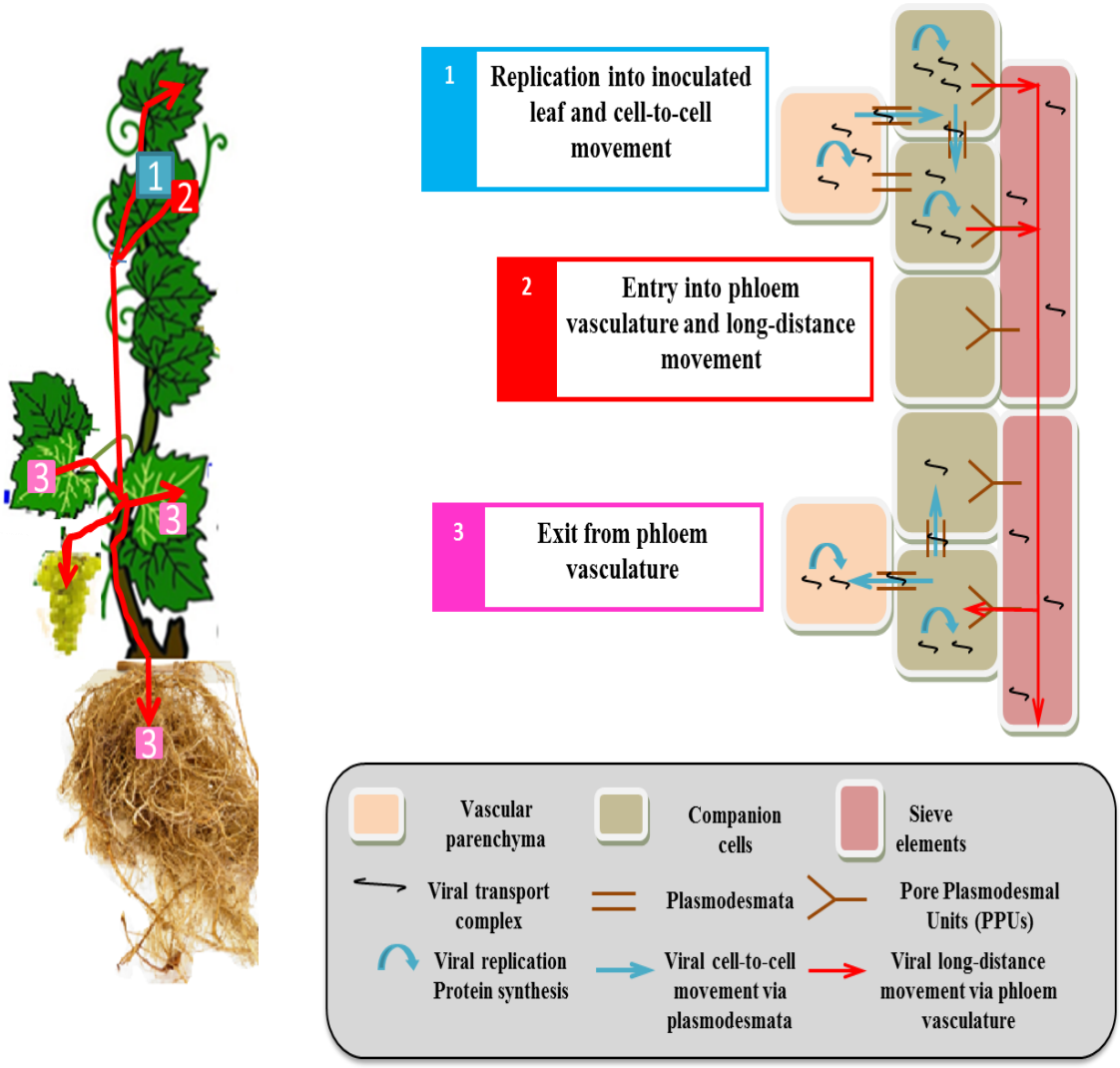

Figure 3. View of virus cell-to-cell and long-distance movement in plant tissues (Modified from Hipper et al. 2013).

3.2. Virus detection of GLRaV-3 and sanitation techniques

Virus detection

Grapevine virus detection can be a difficult task. This is usually because most of the diseased grapevines are infected with more than one virus, or because virus quantity is often very low to be detected. This situation is further complicated because the symptoms can sometimes appear only after two year of infection, viruses are often unevenly distributed in infected vines, and symptoms in some white cultivars and rootstocks are less noticeable.

To date, several techniques have been developed to detect virus associated with GLD in plant material including, biological indexing, serology andnucleic acid-based methods.

Biological indexing

Basically, the Biological indexing method consists for grafting between an indicator grapevine variety, especially sensitive to virus diseases, and scion with subsequent symptoms observation on grafted material. It is one of the most effective and reliable methods used for grapevine leafroll virus detection (Martelli et al. 1993; Martelli and Walter 1998; Pathirana and McKenzie 2005; Constable et al. 2013). Until the late 1980's, biological indexing was the only system for testing GLD. In the traditional biological indexing method, samples to be tested are grafted onto woody indicators grown in soil (Martelli et al. 1993). From 16 months to 3 years is required to complete the indexing procedure and to look after the presence of any virus disease symptoms (Weber et al. 2002).

Green grafting is considered as another biological indexing method (Pathirana & McKenzie 2005). This method consists for grafting green scions or buds onto green shoots. With this method, 80% of the infected grafts show symptoms within 3–4 weeks and 90% within 12 weeks (Walter et al. 2008). This technique is more advantageous than the other system, because it is capable to defeat the graft incompatibility sometimes experienced between distantly related *Vitis* species (Walker and Golino 1999; Walter et al. 2008). Generally, biological indexing is time consuming and labour-expensive and the possible rootstock/scions incompatibility (Weber et al. 2002). In addition, several factors such as virus transmission, the lower virus amount, their uneven distribution in the different organs and environmental grown conditions have been shown to affect the reliability of biological indexing (Rowhani et al. 1997; Constable et al. 2013).

Serological technique

Serological assays were originally developed to detect viruses by utilizing antibodies to detect epitopes of protein antigens. These immunological diagnostic techniques include enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF) and immuno-strip tests (Schaad et al. 2003). Since the 1970s, Enzyme-linked immunosorbent assays are considered the most commonly used immunodiagnostic

technique for virus detection (Clark and Adams 1977; Engvall and Perlmann 1971; Van Weemen and Schuurs 1971). Even if ELISA is not as sensitive as nucleic acid-based techniques, it is more robust, simple and more cost-effective than others, which it makes popular for routing testing to detect GLD in grapevines and to process many samples at the same time

Nucleic acid-based methods

In the recent years, nucleic acid-based methods have increasingly been used to develop diagnostic assays for plant pathogens. It consists to detect the genetic material (ARN or AND) of plant pathogen (Virus or bacterium). These methods have the potential to be very sensitive and highly specific and are based on the unique nucleic acid sequence of the pathogen (Ward et al. 2004; Mothershed and Whitney 2006). Reverse transcription-PCR (RT-PCR) has been used for detection of pathogens such as GLRaV-3 and other variants of this virus. Currently, six genetic variants of GLRaV-3 have been described (Jooste et al. 2010; Gouveia et al. 2011; Wang et al. 2011; Kumar et al. 2012). Due to the genetic variability, multiplex PCRs were developed for the detection of most of the genetic variants of GLRaV-3 (Bester et al. 2012; Chooi et al. 2012). Another procedure to detect GLRaV-3 is the immuno-capture PCR (IC-PCR), which consists in using antibodies, produced against the recombinant major CP, to immobilize GLRaV-3 on the surface of a microfuge tube and then amplify its gens by RT-PCR (Ward et al. 2004; Engel et al. 2008). Additionaly, Spot-PCR is used in woody plants to detect pathogens in a small drop of unbuffered sap from grapevine leaf petiols (Osman and Rowhani, 2006). The Loop-mediated amplification of nucleic acid (LAMP) is an alternative method to PCR, based on the isothermal amplification of a target sequence. GLRaV-3 has been detected by LAMP but introducing reverse transcriptase (RT-LAMP) (Pietersen and Walsh 2012).

The quantification of target DNA has been simplified with the introduction of real-time PCR, in which unknown samples are quantified absolutely or relatively by comparing it to a standard DNA or to a reference gene (Feng et al. 2008). This method requires no post-reaction processing since the amplified product is detected by a built-in fluorometer as it accumulates. Target DNA amplification is detected by using non-specific DNA binding dyes (e.g. SYBR Green) or specific fluorescent probes, like TaqMan chemistry (Ward et al. 2004) (Figure. 4).

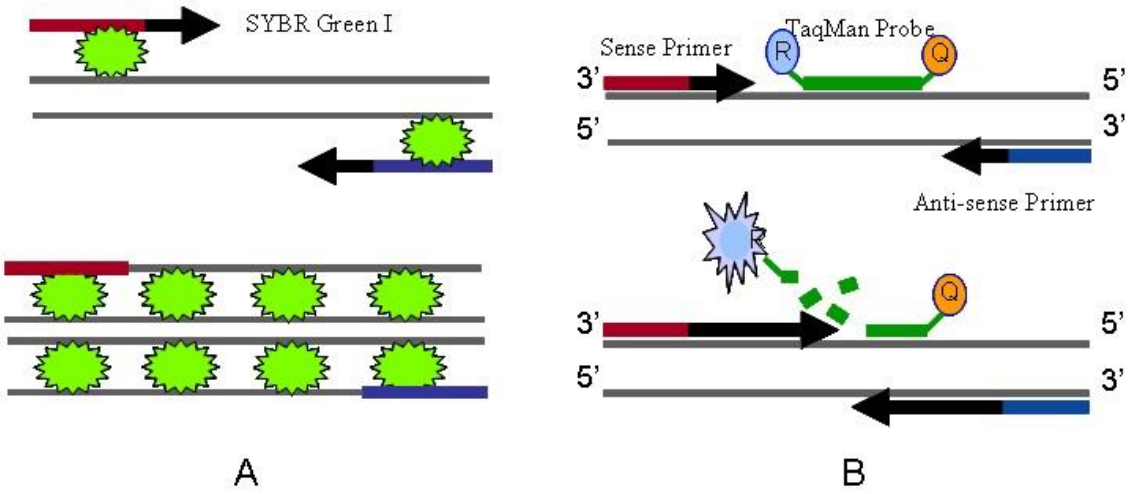

Figure. 4: Two methods used to obtain fluorescent signals from the PCR products. (A) SYBR Green I; (B) TaqMan probes (From Xu et al. 2011).

The use of Real-time TaqMan as a diagnostic tool for the detection of several plant viruses GLRaV-1-5 and 9 has been successfully reported by Osman et al. (2007). Moreover, TaqMan low-density arrays were introduced after a real-time TaqMan assay modification (Osman et al. 2008). Nowadays, Real-time RT-PCR high-resolution melting has helped to identify different genetic variant groups of GLRaV-3. This technique can distinguish changes in a sequence by using DNA binding dye, SYTO 9 (Bester et al. 2012). There are other techniques used for differencing between genetic variants of GLRaV-3, like the single-strand conformation polymorphism (SSCP) profiles and asymmetric PCR-ELISA (APET) (Turturo et al. 2005; Jooste et al. 2010). In this thesis we have choosen to focus on Serological and ARN methods.

Sanitation techniques

The most commonly used sanitation techniques for viruses and viroids are meristem culture, somatic embryogenesis combined or not with thermotherapy and chemotherapy (Panattoni et al. 2013; Parštein et al. 2013; Cheong et al. 2014; Guta et al. 2014). For instance, *in vitro* chemotherapy was used to sanitize plants infected with GFkV (Guta et al. 2014) and to eliminate the *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (Skiada et al. 2013). In order to eliminate GFLV, somatic embryogenesis was used alone (Gambino et al. 2009) or in combination with thermotherapy (Goussard and Wiid 1992). This technique was also used to eliminate GLRaV-1, GLRaV-3, GVA and GRSPaV from three grapevine wine cultivars, Muller-

Thurgau, Grignolino and Bosco (Gambino et al. 2006) and to produce vines free of GLRaV (Goussard et al. 1991), GFkV (Popescu et al. 2003) and ArMV (Borroto-Fernandez et al. 2009). Among the most widely applied methods for virus elimination, a great efficiency was achieved using the combination of meristem or shoot tip culture with thermotherapy (vivo/ vitro) (Milkus et al. 2000; Maliogka et al. 2009; Panattoni and Triolo 2013; Bota et al. 2014). Overall, the effectiveness of virus eradication methods depends on the type and number of virus species that exist in a certain grapevine variety, the cultivar and the protocol used (See chapter 3 for more details).

3.3. Effect of GLRaV-3 on photosyntesis, respiration and carbon balance

Photosynthesis

Grapevine leafroll disease (GLD) induces physiological disturbances in grapevines, mainly associated with photosynthetic impairment. Regardless of the type of the experiment (*in vitro*, pots or in the field), several studies reported a reduction of photosynthetic capacity in GLRaV-3 infected white and red cultivars (Balachandran et al. 1997; González et al. 1997; Christov et al. 2001; Sampol et al. 2003; Petit et al. 2006; Komar et al. 2007; Christov et al. 2007; Basso et al. 2010; Hristov and Abrasheva 2001, Moutinho-Pereira et al. 2012; Gutha et al. 2012 ; Mannini et al. 2012 ; Endeshaw et al. 2014 ; Montero et al. 2016a, 2016b, 2016c). Early studies demonstrated that photosynthetic reduction by single infection (GLRaV-3) or multiple viruses (fanleaf and GLRaV-1, -2 and -3) is associated mainly with non-stomatal limitation. In these studies, photosynthetic decline was associated with low levels of chlorophyll and carotenoid pigments, modifications in the number of chloroplasts, decreases in mesophyll conductance (g_m), inhibition of the activity of the Rubisco and other photosynthetic enzymes, as well as a decrease in the PSII quantum yield (F_V/F_M) (Almási et al. 1996; Hristov and Abrasheva. 2001; Sampol et al. 2003, Bertamini et al. 2004). More recently, it has been shown that leafroll virus infections also caused stomatal limitation in different cultivars such as Touriga Nacional, Cabernet Franc, Malvasia and Giro-Ros (Moutinho-Pereira et al. 2012; Endeshaw et al. 2014; Montero et al. 2016b, 2016c).

These effects are associated with other grapevine physiological disturbances like, sugar transport and accumulation of assimilates, mineral nutrition and hormonal balance processes, which consequently cause the reduction of growth and cropping

(Mannini et al. 1996; Sampol et al. 2003; Moutinho-Pereira et al. 2012, Endeshaw et al. 2014).

Respiration and Carbon balance

Most of the studies have demonstrated the effects of virus infection on photosynthesis, but little is known about its effects on respiration. Plant respiration consumes a great portion of the carbon assimilated by photosynthesis during the day, affecting carbon balance and growth, especially under stress conditions. A recent work shows that GLRaV-3 reduces root and upper-leaf respiration by 36% and 31%, respectively (Montero et al. 2016). Therefore, this reduction has been shown to compensate for the lower photosynthetic carbon assimilation, resulting in an unaffected plant carbon balance in the presence of the virus (PCB). To fight against pathogens, plants protect themselves by the synthesis of defence compounds such as salicylic acid (SA), lignin and phytoalexin. This compounds may account for a respiratory energy and carbon costs (Hanqing et al. 2010). Indeed, it is thought that mitochondria play an important role in stress signaling under pathogen attack, however, litlle is known about mitochondrial metabolism and its control. It is shown that AOX protein and alternative respiratory pathway are frequently induced during plant-virus interaction (Zhang et al. 2012; Cvetkovska and Vanlerberghe 2013).

3.4. Effect of GLRaV-3 on plant hydraulic and water use efficiency

The ability of plants to conduct water from soil to leaves depends on their hydraulic conductance (Meinzer and Grantz 1990; Hubbard et al. 2001; Sperry et al. 2002; Martorell et al. 2014; Martorell et al. 2015). Hydraulic conductance can be measured at leaf, branch or plant level. Water flow through the plant via xylem vessels creates a continuous system from roots to the evaporation sites, which is the so called soil-plant-air continuum. Usually, the conductance is measured as the water flow through a given pathway (leaf, branch, whole plant), divided by the difference of water potential at both ends of that pathway.

The water pathway from the stems of a plant to the evaporation sites in the leaf (leaf hydraulic conductance, K_{leaf}) is critically important for maintaining a correct leaf

water balance. Indeed, most of carbon assimilation and water losses take place in the leaves, which represents an important limitation for the hydraulic system with an average resistance of the total plant of up to 30%, even in some cases it can account for the 80% (Sack and Holbrook 2006). Under water stress conditions, K_{leaf} has been showed to be severely limited (Salleo et al. 2001; Johnson et al. 2011; Bucci et al. 2012), but this change may also occur when tree tissues are invaded by pathogens such as bacteria and fungi (Talboys 1968; Dimond 1970). From our best knowledge, there is very little work on the influence of GLRaV-3 on K_{leaf}. Two studies have showed the different effects of this specific phloem limited virus on water flow, one in grapefruit (Moreshet et al. 1998) and another in grapevine (Pantaleo et al. 2016). The first study revealed that after six years inoculation with citrus viroid (CVd), grapefruit showed reductions in leaf and root hydraulic conductance and less water uptake per unit (Moreshet et al., 1998). Nevertheless, Pantaleo et al. (2016) reported that the presence of *Grapevine rupestris stem pitting associated virus* (GRSPaV) in white grape cultivar Bosco (*V. vinifera* L.), increased significantly stomatal conductance and stem+shoot hydraulic conductance as compared with GRSPaV-free plants.

Reducing water use for irrigation and increasing water use efficiency (WUE as the yield to water consumption ratio) has become a major priority in agricultural and viticulture research (Morison et al. 2008). In light of the pressure imposed by climate change, several strategies will be required to improve WUE by plant breeding (Cattivelli et al. 2008), but the effects of plant pathogens have not been considered adequately in this context. WUE can be measured at different levels (crop, plant or leaf level) and at different time scales, from months to minutes (Morison et al. 2008; Medrano et al. 2015). At leaf level, it is common to use single-leaf gas exchange measurements, relating net CO_2 assimilation rate (A_N) either to stomatal conductance (g_s), designated intrinsic WUE_i (A_N/g_s) or to leaf transpiration rate (E), defined as instantaneous WUE_{int} (A_N/E). The carbon isotope ratio ($\delta^{13}C$) of leaf dry matter is often viewed as an indicator of long-term WUE at the leaf level (Farquhar and Richards 1984). Whole-plant WUE (WUE_{WP}) reflects the actual WUE, at a larger spatial (whole plant) and temporal scale (whole growth period) than leaf-level estimates. WUE_{WP} depends on physiological processes developed at the leaf level: photosynthesis, respiration and transpiration (Flexas et al. 2010). Thus, deleterious effects on WUEint (A_N/E) have been demonstrated in a number of pathosystems (Grimmer et al. 2012), as

for the fungi *Uncinula nectar* (Powdery Mildew) affecting grape leaves (Lakso et al. 1982). Further studies have also identified the reduction of WUEi by fungal diseases of pecan (Andersen et al. 1990) and common bean (Jesus Junior et al. 2001) using leaf gas exchange equipment. Furthermore, previous studies showed that reduced transpiration in host plants was associated with the number of viral infections (Gondo M 1953; Lindsey et al. 1974). However, the effect of GLRaV-3 in grapevines (*Vitis vinifera* L.) water use efficiency are far to be understood. Recently, it has been shown that Grapevine leaf-roll associated virus (GLRaV-1 and GLRaV-3) increased the intrinsic water use efficiency (A_N/g_s) in Touriga Nacional cv. under filed conditions (Moutinho-Pereira et al. 2012). In this sense, the effect of this virus in WUE_{leaf} and WUE_{wp} is a core subject of interest for a sustainable viticulture.

4. COMBINATION OF ABIOTIC AND BIOTIC STRESSES ON GRAPEVINE PHYSIOLOGY

Under natural conditions, plants are challenged by a wide range of environmental changes, leading them to develop mechanisms to tolerate extreme situations. Plants are often subjected to a number of abiotic and biotic stresses, which are often tolerated individually by the plants, however, when two or more of these stresses are expressed simultaneously, plants may lose their bearings (Atkinson and Urwin 2012; Mittler 2006; 2010; Nostar et al. 2013). According to Carvalho et al (2015), the plants affected by a combination of stresses have been triggering synergistic or antagonistic physiological, metabolic or transcriptomic responses.

Within the context of the climate change, the occurrence of combined drought and heat stresses, as well as drought and temperature are perhaps the most environmental factors limiting plants growth and yield in agriculture areas worldwide (Suzuki el al. 2014), suggesting that they reduce average yields by >50% for most major crop plants (Wang et al. 2003).In addition, evidences also suggest that the climate change will also expand the host range of pathogens with increased chances of virulent strain development (Garrett et al. 2006). Unlike other abiotic and biotic stresses combinations, water stress and pathogen were considered one of the most studied interactions in some crops (Carter et al. 2009; Ramegowda et al. 2013; Sharma et al. 2007; Xu et al. 2008). Regarding the effect of combined stress on water transport and

water use, there are few works that discuss about the idea that water stress enhances symptom severity along the stem in plants infected with xylem limited bacteria *Xylella fastidiosa* (Xf) (McElrone et al. 2001, 2003). However, other works pointed out the idea that interaction of plants with pathogens can also be beneficial to improve abiotic stress tolerance (Xu et al. 2008; Reusche et al. 2012). For instance, infection with the vascular pathogen *Verticillium* spp. increased *Arabidopsis thaliana* drought tolerance due to *de novo* xylem formation, which enhances water flow (Reusche et al. 2012).

Nevertheless, the co-occurrence of such stress combinations has been poorly investigated at the moment in grapevines. In Pantaleo et al. (2016), the interaction between GRSPaV and *V. Vinifera* cv. Bosco seems to improve the drought tolerance of infected grapevines in greenhouse conditions. Surprisingly, the infected plants in this study showed a higher photosynthetic rate, stomatal conductance and hydraulic resistance to water transport when compared to healthy plants under water stress conditions. The effect of virus on grapevine physiology and their response to environmental conditions has only recently emerged a high interest in the filed of viticulture. Thus, in the present Thesis we aimed to underline the effect of virus, water stress and its interaction on grapevine physiology.

OBJECTIVES

The general objective of the present thesis is to study the effects of virus infection and moderate water stress and its combination on main physiological processes in two local grapevine cultivars. The study of sanitary status and the identification of the main limitations to grapevine performance under GLRaV-3 infection and moderate water stress can contribute to the milestone of conservation and incorporation of these cultivars to the Mediterranean viticulture.

This general aim is divided in four specific objectives. Each of this objectives are mainly adressed into one of the chapters and corresponded to on of the publications derived from the present thesis:

1. To study the sanitary status of Majorcan minority grapevines cultivars and to highlight the prevalence of *Grapevine leafroll associated virus-3* (GLRaV-3) in local cultivars.

"El Aou-ouad H., Montero M., Baraza E. & Bota J. (2017). Sanitary status of majorcan local grapevines cultivars and elimination of multiple infections from two *Vitis vinifera* cultivars combining thermotherapy with shoot tip culture. *European Journal of Plant Pathology* (submitted)**."**

2. To determine the effects of water deficit, GLRaV-3 infection and its combination on plant growth and primary metabolism.

"El Aou-ouad., Florez-Sarasa I., Obata T., Montero R., Fernie A.R., Medrano H., Pou A. & Bota J. (2017). Physiological and metabolic changes in grapevines under combined drought stress and virus infection. *Frontier in Plant Science* (submitted)**."**

3. To determine the effects of water deficit, GLRaV-3 infection and its combination on photosynthesis identifying which part of the photosynthetic machinery was mainly affected (diffusion or biochemical limitations).

"El Aou-ouad H., Montero R., Medrano H., Bota J. (2016). Interactive effects of grapevine leafroll-associated virus 3 (GLRaV-3) and water stress on the physiology of *Vitis vinifera* L. cv. Malvasia de Banyalbufar and Giro-Ros. *Journal of Plant Physiology*, **196**: 106- 115.**"**

4. To investigate the effect of water deficit, GLRaV-3 infection and the combination of both stresses on on hydraulic conductance and consequences on water use efficiency at leaf and plant levels.

"El Aou-ouad H., Pou A., Tomàs M., Montero R., Ribas-Carbó M., Medrano H. & Bota J. (2017). Combined effect of virus infection and water stress on water flow and water economy in grapevines. *Physiologia Plantarum.* DOI: 10.1111/ppl.12541**."**

Chapter 1

SANITARY STATUS OF MAJORCAN LOCAL GRAPEVINES CULTIVARS AND ELIMINATION OF MULTIPLE INFECTIONS FROM TWO *VITIS VINIFERA* CULTIVARS COMBINING THERMOTHERAPY WITH SHOOT TIP CULTURE

SANITARY STATUS OF MAJORCAN LOCAL GRAPEVINES CULTIVARS AND ELIMINATION OF MULTIPLE INFECTIONS FROM TWO *VITIS VINIFERA* CULTIVARS COMBINING THERMOTHERAPY WITH SHOOT TIP CULTURE

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ABSTRACT

Nowadays, recuperation and genetic diversity preservation of local cultivars have acquired a huge interest in viticulture areas worldwide. In the Balearic Islands, most of the old cultivars are only preserved in grapevine germplasm banks and so far the sanitary status of these local cultivars has remained unexplored, leading to deterioration and loss of certain cultivars in this area. The aim of this study was to survey and detect the virus incidence of all conserved cultivars in the germplasm collection of the Balearic Islands and to promote the sanitary recover of two important minor cultivars, Argamussa and Gorgollassa, through an effective sanitation protocol to eliminate simple and multiple virus infections.

Enzyme-Linked Immuno-Sorbent Assay (ELISA) screenings were performed in 315 vines of 33 local cultivars. It has been shown that local cultivars were highly infected with single (39. 68%) and mixed infection (52. 07 %) and only 8.25 % of the plants tested were considered virus-free. *Grapevine leafroll associated virus-3* (GLRaV-3) infection was the most common (82%). Moreover, *Grapevine fanleaf virus* (GFLV) and *Grapevine fleck virus* (GFkV) were also present with considerable incidence, 25.39 % and 43.49 %, respectively. In addition, we have been used two described sanitation protocols for double and triple viruses' eradication: Shoot tips culture (ST) and thermotherapy in combination with shoot tips culture (CT). Virus elimination using only ST was effective to obtain Argamussa and Gorgollassa cvs. virus-free plants. In addition, it is important to emphasize that the two used methods described in the current study were rapid and effective to eliminate GLRaV-3, the most common grapevine virus worldwide, as well as GFLV.

Key words: Grapevine local cultivars recuperation, virus incidence, sanitation techniques.

INTRODUCTION

Due to several factors, the grapevine genetic diversity gained, over the millennia, is being drastically reduced all over the world (This et al., 2006). At the end of 19th century, different diseases from America reached Europe (Powdery mildews, Phylloxera (*Phylloxera vastatrix*)) causing a huge devastation of many European vineyards, which was reflected in drastic changes in the diversity of this species (cultivated and wild grapes). Moreover, over the last 50 years, cultivated grapevines had undergone another drastic reduction owing to the impact of globalization of wine and quality demarcation of a number of cultivars and vineyard areas. In fact, the emergence of the few cultivars grown worldwide such as Chardonnay, Cabernet Sauvignon, Syrah (Shiraz) and Merlot are increasing and causing at the same time the disappearance of old local cultivars, that considered perfectly adapted to the local environmental condition (Cipriani et al., 2010; Terral et al., 2010). The sanitary selection of disease-free clones has also induced a reduction in the clonal diversity of these major cultivars around the world. Currently, recuperation and preservation of the genetic diversity of local cultivars are considered a challenge in viticulture area worldwide, and it becomes a necessity to fight against the general process of genetic erosion. In order to limit this loss of genes and genotypes, nearly every wine growing area has preserved plants in germplasm banks (This et al., 2006; Maghradze et al., 2010). Grapevine diversity reduction, due to the factors listed before, has been shown to be more pronounced in isolated area as Balearic Islands (García-Muñoz 2012). Even thought, and despite of their small geographic area, the grapevine diversity found in the Balearic Islands is considered very high (García-Muñoz 2011). The Balearic wines were well known worldwide for their high quality since Roman times (Hidalgo 2002). More recently, several studies have been shown the high enological aptitude of some minor cultivars from these islands (Escalona et al., 2009, 2012; Bota et al., 2013). As reported by García-Muñoz et al. (2014) the sensorial analysis of 18 minor varieties wines revealed the acceptation of the quality of these wines in a similar way as the varieties included in Designation of Origin (DO). Today, the wine consumers eager to try new products included in the (DO), thus the use of minor varieties could be a paramount factor to fulfill this gap and to satisfy DO requirements (Santiago et al., 2008).

In spite of its importance, the sanitary status of local cultivars has remained neglected and unexplored, leading to deterioration and loss of certain cultivars in the Mediterranean areas and other countries (Komínek and Holleinová 2003; Cretazzo 2010; Bota et al., 2013; Salami et al., 2009; Mahfoudhi et al., 2014). Remarkably, the surveys of several grape-growing regions have revealed the high prevalence of the most widespread virus diseases, *Grapevine Leafroll Disease* (GLD), *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV) and *Rugose Wood* (RW) (MacKenzie et al., 1996; Digiaro et al., 2000; Peršurić et al., 2011; Maree et al., 2013; Mahfoudhi et al., 2014; Sharma et al., 2015). It has been shown that the local grapevine varieties usually present a very high incidence of virus infections (Poljuha et al., 2004; Materazzi et al., 2006; Zdunic et al., 2007). Particularly, in the Balearic Islands, Cretazzo et al. (2010) research has shown that three Majorcan autochthonous grapevine cultivars (Manto Negro, Callet and Moll) were highly infected by *Grapevine leafroll associated virus* (GLRaVs). In fact, only 6.4%, 9.6% and 11.5% of Manto Negro, Callet and Moll, respectively were virus-free. This viral infection can strongly compromise the clonal selection and the authorization of the cultivars, since the EU Directive 2005/43/EC regulations require that the initial plant material for vegetative propagation is free of *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine fleck virus* (GFkV, only for rootstocks), *Grapevine leafroll associated virus-1* (GLRaV-1), and *Grapevine leafroll associated virus- 3* (GLRaV-3). Besides, it has been shown that these viruses are able to change leaf morphology, performance and the ampelographic features of the selected vines (Mannini 2000). The high incidence of virus diseases could be explained by several causes (Bertolini et al., 2010; Le Maguet et al., 2012; Bertin et al., 2016), whereas the presence of these viruses in the coastal regions could mainly attributed to vector-borne transmission (Cabaleiro et al., 2008; Sharma et al., 2015).

It is well documented that viral infections reduce the productive life of plants and provoke severe reductions in yield and quality (Andret-Link et al., 2004; Padilla et al., 2007; Naidu et al., 2014; Montero et al., 2016a). Therefore, it becomes crucial to know the prevalence and distribution of grapevine viruses to create appropriate sanitary measures to preserve our genetic resources and to propagate healthy material. In endemic areas, sanitation becomes the main focus of the clonal selection process; because once a vine is infected, there is no cure. The most commonly used sanitation

techniques for viruses and viroids are meristem culture, somatic embryogenesis combined or not with thermotherapy and chemotherapy, showing differential success according to viral species (Panattoni and Triolo 2013; Guta et al., 2014; Maliogka et al., 2015). For instance, *in vitro* chemotherapy was used to sanitize plants infected with GFkV (Guta et al., 2014) and with *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (Skiada et al., 2013). In order to eliminate GFLV, somatic embryogenesis was used alone (Gambino et al., 2009) or in combination with thermotherapy (Goussard and Wiid 1992). This technique was also used to eliminate GLRaV-1, GLRaV-3, GVA and GRSPaV from three grapevine wine cultivars, Muller-Thurgau, Grignolino and Bosco (Gambino et al., 2006) and to produce vines free of GLRaV (Goussard et al., 1991), GFkV (Popescu et al., 2003) and ArMV (Borroto-Fernandez et al., 2009). One of the best methods to eliminate GFkV from Manto Negro cv., is using the combination of meristem or shoot tip culture with thermotherapy (in vivo/ in vitro culture) (Bota et al., 2014). Heat treatment above 35°C has been reported to impede virus replication, followed by nucleic acid phosphodiester covalent bonds and consequently enhances its disorganization and deterioration of viral infectivity, thus leading to the eradication of the virus from the shoot tips (Cooper and Walkey 1978; Panattoni and Triolo 2013). Whereas the number of infected plants is very high among local cultivars, quick and easy methods should be sought in order to sanitize a large amounts of material.

The aims of this work are: (i) to elucidate the current sanitary status of the local cultivars conserved in the germplasm collection of the Balearic Islands and (ii) to explore the effectiveness of two sanitation methods used within two well-known minor cultivars from this region (Argamussa and Gorgollassa) in order to obtain free-virus plants and to get new licensable material to be commercialized.

MATERIAL AND METHODS

Incidence of virus infection

Plant material

The study was conducted in 315 plants corresponding to 33 traditional cultivars from the Balearic Islands (Spain) which are conserved in the Germplasm Collection (GCPM) located at the experimental station of Sa Granja in Palma de Mallorca. The

plants were 10-15 years old and grafted on 99-Richter rootstock. Between 3 to 18 replicates per cultivar were collected between 2009 and 2014 in order to assess their sanitary status. All the material of the collection was previously characterized by combining ampelography, microsatellite analysis and synthesis of historical references of the cultivars (Garcia-Munõz et al., 2012).

Serological tests

In this study, we tested the most harmful grapevine viruses according to the Commission Directive 2005/43/EC amending the Annexes to Council Directive 68/193/EEC on the marketing of grapevine propagation material. *Grapevine leafroll associated virus* (GLRaV-1, 2 and 3), *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV) and *Grapevine fleck virus* (GFkV) were tested in each plant of the 33 cultivars by enzyme-linked immunosorbent assay (ELISA) (Clark and Adams 1977) using commercial coating and conjugate antibody preparations (Bioreba AG, Reinach, Switzerland). In order to obtain as much confidential results as possible, and to minimize false negative results due to uneven distribution of viruses in plants, the collected samples from each investigated plant was taken from the appropriate tissue, at the right time of year, and in good physical conditions as it is described in (Padilla 2009). The samples were labeled, placed in plastic bags and stored at 4ºC before testing, which was completed within the limit of one week after collection. For GFkV detection, the sampling was performed in spring (April-May). Each sample consisted of five shoot basal leaves picked around the vine perimeter. In case of GLRaV-1,-3, GFLV and ArMV, collection was performed in October. For each leaf, the terminal part of the petiole and the contiguous portion of limb were excised for the extraction. Samples were considered 'positive' when the values of absorbance at 405 nm were at least two times higher than the two negative controls (healthy grapevines) included in the same plate.

Sanitation of infected material

Plant material

Two minor grapevine cultivars were used for sanitation procedure, the white cultivar Argamussa and the red cultivar Gorgollassa. Elisa test was used to evaluate the presence of the following virus in mother plants and also in plants after sanitation

treatments: GFLV, GLRaV-1, GLRaV-3, GLRaV-4-9 (Grapevine leafroll-associated virus generic 4 strains; in this case, the "generic reagents" recognize GLRaV-4, GLRaV-4 strain 5, GLRaV-4 strain 6, GLRaV-4 strain 9), ArMV and GFkV. Samples collection and virus testing were performed as described above for incidence of virus.
After sanitation procedures, the absence of viruses was confirmed using RT-PCR technique. The presence of GFLV, GLRaV-1, GLRaV-3, and GFkV was analyzed using specific primers (Osman et al., 2008). Unfortunately, RT-PCR was performed neither in GLRaV-2 nor in GLRaV-4-9. Total RNA was extracted from 50 mg of phloem scraped from mature canes or leaves by using Spectrum Plant Total RNA Kit (Sigma-Aldrich) according to manufacturer's instructions. The Spectrum™ Plant total RNA Kit removed most of the DNA during RNA purification. RNA purity and concentration were measured at 260/280 nm using a spectrophotometer (NanoDrop-1000, Thermo Scientific, Villebon sur Yvette, France). First-strand cDNA synthesis with final volume of 20 µl was performed using 500 ng of total RNA, 200 units of recombinant Moloney Murine Leukemia Virus (MuLV) reverse transcriptase (Invitrogen Life Technologies, Inc.), 40 units of RNase inhibitor (RNase out, Invitrogen Life Technologies, Inc.), 0.4 mM of dNTPs, and 2 mM of random nonamers (Takara Bio, Inc.). The mixture for reverse transcription (20 µl) was incubated for 50 min at 37ºC and the reaction was inactivated by heating it at 70ºC for 15 min.

Real-time PCR analysis was performed using 2 µl of diluted (1:100) cDNA in 25 µl of reaction medium containing, 1 mM of dNTPs, 0.5 mM of each primer and 5 units of Taq polymerase (Takara TaqTM, Takara Bio). Thermo-cycling was performed as follows: 30 min at 52 ºC followed by 35 cycles of 94 ºC for 30 s, 58 ºC for 45 s and 72 ºC for 60 s, final extension at 72 ºC for 7 min, and storage at -20 ºC. Finally, 10 ml of amplification product was electrophoresed on a 2 % agarose gel in TBE buffer [90 mM Tris–borate, 2 mM EDTA, pH (8.0)], stained with ethidium bromide, visualized on an UV transilluminator and photographed. Positive and negative samples of each virus were included as controls in each test.

Sanitation procedure

For Gorgollassa and Argamussa, two sanitation methodologies were compared to determine their effectiveness of the latter two grape varieties: shoot tip culture (SC) and chamber thermotherapy and shoot tip culture (CT).

The plants used in both methodologies were obtained by direct rooting of 0.2-m dormant canes selected from mother plants from GCPM. Plants were maintained in the greenhouse until sprouting. A total of 67 plants were used in case of Argamussa and 300 plants in case of Gorgollassa. After 6-7 weeks, half of the plants were used for SC treatment and the others for CT treatment. CT treatment consisted in gradually increasing the growth temperature by 4 ºC per week going from 26 ºC/22 ºC to 37.5 ºC/34.0 ºC (day/night). These conditions were maintained for 40 days on a 16-h photoperiod at a light intensity of 56 μmol m^{-2} s^{-1} provided by white fluorescent tubes.
In both methodologies, plants with actively growing shoots were stripped of leaves and washed with tap water. Single node segments were disinfected using 70% Ethanol for 40s and soaked in an aqueous solution of sodium hypochlorite 10% with few drops of Tween 20 for 15 min, shaken and rinsed three times with sterile distilled water to remove sterilizing agents.

After surface sterilization, shoot tips (1-3 mm) were isolated and cultivated on half-strength Murashige and Skoog's basal medium (Murashige and Skoog 1962), 2 % (w/v) sucrose and agar 0.7 % supplemented with 2.25 mg/L of 6-benzyladenine (MS-BAP), pH was adjusted to 5.7 prior to autoclaving. Glass test tubes (150 mm x 24 mm) with 25- mm diameter plastic stoppers (Kap-Uts K25, Bellco) containing 20 ml of medium were used. Cultures were incubated in a Fitoclima S600PLH chamber (Aralab) at 23 ºC under a 16-h photoperiod and light intensity of 56 μmol m^{-2} s^{-1} provided by white fluorescent tubes (LUMILUX Cool White, L18W/840-Osram). Relative humidity (RH) was around 60%. After 6-8 weeks of culture, explants were transferred to rooting media containing basal medium (MS) with half strength of macro and microelements.

Transfer to *ex vitro* conditions

Rooted plantlets measuring 3-5 cm were transferred to sterile soil and maintained in a growth chamber at 25 ºC, under low light intensity and 90 % RH for 15 days. Pots were enclosed in clear polyethylene bags to minimize moisture loss and keep culture cabinet conditions. Plantlets were transferred into a greenhouse under higher light intensity but still with high RH (70-80%). RH was gradually decreased to obtain full *ex vitro* acclimation and active growth. Two months later, plants were placed outdoors under a shading mesh to protect them from direct sunlight. Plants were grown outdoors in 2-L pots filled with organic substrate and perlite mixture (2:1).

Approximately one year after acclimation, when plants had sufficient lignified material, plants were tested for virus presence.

Statistical analyses

To analyze the differences on the sanitary status among cultivars, a nominal logistic model was used with the identity of the virus presented as response variable. To compare the effect of sanitation treatments on virus eradication in Gorgollassa plants, generalized linear models (GLM) with binomial distribution are fit using JMP 10 software package.

RESULTS

Incidence of virus infection

The results of serological analysis revealed that the local varieties were infected with all the viruses tested in this study (GLRaV-3, GLRaV-1, GFLV and GFkV), except for Arabis mosaic virus (ArMV).

In general, high infection rates were obtained for all the studied cultivars (Fig. 1). Only 8.25 % of the tested plants were virus-free and multiple infections were very common (52.07 %; Fig. 1). In the 33 cultivars studied, the GLRaV-3 was the most predominant virus, alone or combined with other viral disease. Therefore, its incidence is the highest, reaching 82 % of total plants. The second most frequent virus in the collection was GFkV, occurring in 24 local cultivars with a total incidence of 43.49 %. GFLV was found in 14 local cultivars with a total incidence of 25.39 %. Finally, GLRaV-1 was the least common, occurring in 4 cultivars, with only 3.8 % of total incidence (Fig. 1).

The percentage of plants showing simple infection with any of the viruses' studies was 39.68 %. In these plants, the most common virus infection was GLRaV-3, with its incidence reached up 80 % of the single infections being present in 20 cultivars (ranging from 6 % -100 %) (Table 1). In this collection the most frequent double infections were GLRaV-3 + GFkV (71.43 % in 17 cultivars) and GLRaV-3 + GFLV (25.21 % in 9 cultivars). Single infection with GFLV was very low, since this virus has

been found often combined with GLRaVs and GFKV in local cultivars (Fig.1). Furthermore, in plants with triple infection, the most common mixed infection was (GFLV + GFkV + GLRaV-3) with incidence of 92.86 %, of the total triple infection, in 9 cultivars. Other multiple infections in the collection were less common, with incidences of less than 4 % (Fig.1 Table 1).

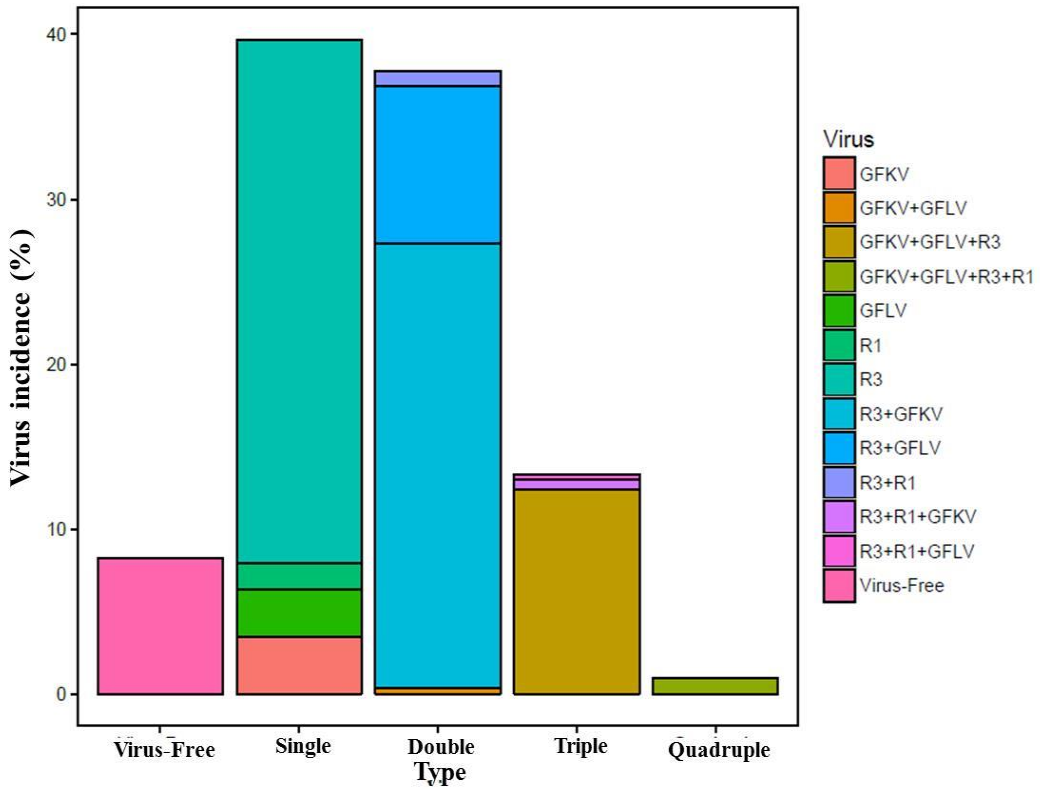

Figure 1: Single and multiple virus incidences per variety (in %). R1: *Grapevine leafroll associated virus*-1 (GLRaV-1), R3: *Grapevine leafroll associated virus*-3 (GLRaV-3). GFkV: *Grapevine fleck virus*, GFLV: *Grapevine fanleaf virus.*

The incidence and distribution of these viruses is unequal among the cultivars (L-R χ^2=718.73; P<0.0001 nominal logistic model). Details of sanitary status of all plants include in the collections were explained in Supplementary Table S1 and summarized in Table 1. Only in 9 of the 34 cultivars virus-free plants were found. Malvasia de Banyalbufar, Girò-Ros, Gorgollassa and Mancès de Tibus are the varieties with the highest number of healthy individuals (Table 1). In some varieties, all the plants analyzed have the same type of infection, such as Argamussa, Calop Blanc, Calop Negre and Calop Roig 100% infected with GLRaV-3 and GFkV, Magdalena with 100% of GLRaV-3 infection or Esperò de Gall with 88.8% of triple infection.

The occurrence of GLRaV-3 (single or combined) ranged between 6 % in Vinater Tinto and 100 % in other 18 cultivars (Table 1). Only six cultivars, Callet Negrella, Mandó, Vinater tinto, Manto Negro, Mances de Capdell and Fernandella, presented GFkV, while simple infection with GFLV was found only in Gorgollassa (50%) and Vinater tinto (11.76 %) and GLRaV-1 was presented only in Malvasia de Banyalbufar (31.25 %).

Sanitation of Gorgollassa and Argamussa local cultivars

Argamussa mother plants were infected with GLRaV-3 and GFkV, displaying single and double infections. Gorgollassa showed double (GFLV, GLRaV-4-9) and triple (GLRaV-3, GFLV, GLRaV-4-9) infections.

In case of Gorgollassa cv., both sanitation treatments, SC and CT, have been proved to be equally efficient to eliminate virus infection (no significant differences in infection incidence between treatments L-R χ^2=0.43; P=0.51 GLM binomial). Since the percentage of sanitized plants, was not significantly increased when the heat treatment was applied, is being the sanitation rate similar using SC (76.9 %) and CT (86.66%). After sanitation, the plants that remained infected showed only the presence of GLRaV-4-9.

Unfortunately, in case of Argamussa cv., it was impossible to evaluate the results of CT treatment, since no survival plantlets were obtained. Shoot tips culture alone resulted in only one GLRaV-3+GFkV-free plant (16.67%) while 66.67% of plantlets resulted GLRaV-3-free but still infected with GFkV.

Table 1: Healthy plants (No-infected), single, double and multiple virus incidences per cultivar (% of total plants)

Local cultivars	No infected	Simple infection (%)	Double infection (%)	Multiple infections (%)
Argamussa	0	0	100.0 (R3+GFkV)	0
Batista Felanix	0	77.7 (R3)	22.2 (R3+GFkV)	
Callet	0	77.7 (R3)	22.2 (R3+GFkV)	
Callet Negrella	0	57.1 (R3) / 67(GFkV)	14 (R3+GFkV)	0
Calop Blanc	0	0	100 (R3+GFkV)	
Calop Negre	0	0	100 (R3+GFkV)	
Calop Roig	0	0	100 (R3+GFkV)	
Escursac	30	40 (R3)	30 (R3+GFLV)	
Esperó de Gall	0	0	11 (R3+GFkV)	88.8 (R3+GFkV+GFLV)
Fernandella	37.5	37.5 (R3)/ 12.5 (GFkV)	12.5 (R3+GFkV)	
Fogoneu	0	77.7 (R3)	11.1 (R3+GFLV)	
Gafarro	14.3	85.7 (R3)	0	
Galmeter	0	0	0	85.7 (R3+GFkV+GFLV) 14.2 (R3+R1+GFLV+GFkV)
Giró Ros	60	40.0 (R3)	0	
Gorgollassa	10	50.0 (GFLV)	40.0 (R3+GFLV)	
Jaumes	12.5	12.5 (R3)	50.0 (R3+GFLV)	25.0 (R3+GFkV+GFLV)
Magdalena	0	100.0 (R3)	0	
Malvasia de B.	31.2	12.5 (R3)/ 31.2 (R1)	18.7 (R3+R1)	6.2 (R3+GFkV+GFLV)
Mamella Vaca	0	100 (R3)	0	
Mances de Capdell	0	44.4 (R3)/5.5 (GFkV)	5.5 (R3+GFkV)/5.5 (R3+GFLV)	38.8 (R3+GFkV+GFLV)
Mancés de Tibus	70	10 (R3)/ 20.0 (GFLV)	0	
Mandó	0	62.5 (GFkV)	37.5 (R3+GFkV)	
Manto negro	0	55.5 (R3)/ 11.1 (GFkV)	33.3 (R3+GFkV)	
Mateu	0	100 (R3)	0	
Molinera	0	100 (R3)	0	
Moll	0	33.3 (R3)	66.6 (R3+GFkV)	
Quigat	0	0	87.5 (R3+GFkV)	12.5 (R3+R1+GFkV)
Sabater	0	0	77.7 (R3+GFLV)	11.1 (R3+R1+GFLV) 11.1 (R3+GFkV+GFLV)
Sinso	0	78 (R3)	22 (R3+GFLV)	
Valent Blanc	0	0	33.3 (R3+GFLV)	50.0 (R3+GFkV+GFLV) 8.3 (R3+R1+GFLV) 8.3 (R3+R1+GFLV+GFkV)
Valent Negre	0	0	22.2 (R3+GFkV)	66.6 (R3+GFkV+GFLV) 11.1 (R3+R1+GFLV+GFkV)
Vinater Blanc	0	0	100 (R3+GFkV)	
Vinater Tinto	0	5.8 (R3) 5.8 (GFkV) 11.7 (GFLV)	23.5 (R3+GFLV) 35.2 (R3+GFkV) 5.8 (GFkV+GFLV)	11.7 (R3+GFkV+GFLV)

R1: *Grapevine leafroll associated virus*-1 (GLRaV-1), R3: *Grapevine leafroll associated virus*-3 (GLRaV-3).

DISCUSSION

The results of the current study, conducted in 33 grapevine cultivars from the Germplasms bank of the Balearic Islands, revealed a high presence of the most widespread viruses in different levels of incidence. Nevertheless, the test for Arabic mosaic virus (ArMV) resulted negative for all the samples tested. These results have been expected because it was noted for this specific virus that its prevalence in Spain was very low (Abelleira et al., 2010) and never has been found in Baleares.

In the current study, the high prevalence of GLRaV-3 (82%) found in minor varieties was higher than the one found in the three major local varieties cultivated (70%) in Mallorca (Manto Negro, Callet and Moll) (Cretazzo et al., 2010). Several studies have been shown that GLRaV-3 is also fairly widespread in Spain (Padilla et al., 2009, 2012, Cabaleiro et al., 2008) as well as in several Mediterranean viticulture areas (Digiaro et al., 2000; Savino et al., 2001; Mahfoudhi et al., 2014) and in the rest of the world (MacKenzie et al., 1996; Reynard and Gugerli 2012; Maree et al., 2013; Sharma et al., 2015). For instance, high incidence of GLRaV-3 was also determined for 139 natives' cultivars from Croatia collection, with incidence rates varying from 72 to 100% (Voncina et al., 2011), and for autochthonous Istrian cultivars (61.1%) (Peršurić et al., 2012). One possible explanation of the high incidence of GLRaV-3 with respect to other GLRaVs could be its higher multiplication efficiency in the host as reported by Velasco et al. (2014). The high spread of GLRaV-3 in our collection is most likely related to the presence of virus vector (Mealybug). There is clear evidence of the presence of *Planococcus citri* in vineyards of the Balearic Islands (Conselleria d'Agricultura i Pesca, Govern de Les Illes Balears, personal communication), and this insect is a well-known vector transmission of GLRaV-3 (Cabaleiro et al., 2008). Other studies have been proved that in the coastal regions the distribution of GLRaV-3 was commonly associated to the Mealybug infestations (Taylor et al., 2015; Sharma et al., 2015). Because, Mealybug prefer mild warm temperature and high humidity (Cornwell 1958; Grasswitz and James 2008).

Similarly as Cretazzo et al. (2010), our results revealed that the presence of GFLV and GFkV was also high in the local cultivars, being 40% and 70%, respectively. In most cultivars, GFLV was combined with GLRaV-3, while the double infection with

GFLV+ GFkV was observed only in Vinater tinto cultivar. The viruses' distribution in our study was consistent with previous works conducted in local Istrian (Croatia) varieties (Poljuha et al., 2004, 2010), being the distribution of GFLV was lower (14-23.9%) than GLRaV-3 (69.1-72.3%) and GLRaV-1 (17.2-24.3%). In addition, the incidence of GFLV presented in our study was higher than that reported for Croatian local cultivars (11 %) (Zdunić et al., 2008) and lower than what was reported in different areas of Iran (Salami et al., 2009).

Remarkably, our results showed that the incidence of GFkV was higher than for GFLV; being this virus generally combined with GLRaVs. The high incidence of GFkV was also observed in other growing area such as, Andalucía (Spain), Istria (Croatia), Iran and Chile (Akbas et al., 2007; Fiore et al., 2008; Kumar et al., 2013; Velasco et al., 2015). In some viticultural region, GFkV was the most widespread as compared to GLRaV-3, GLRaV-1 and GFLV (Dida et al., 2012; Megrelishvili et al., 2016). GFkV is latent in *Vitis vinifera* and many grapevine varieties and rootstocks infected by this virus are symptomless. The European certification regulations require the absence of GFkV only in rootstocks and not in *Vitis vinifera* L. (Commission Directive 2005/43/EC amending the Annexes to Council Directive 68/193/EEC). However, the presence of GFkV can affect physiological processes (Bota et al., 2014). The high incidence of GFkV reported in this study pointed out the importance of this virus in different cultivars of grapevine, concluding that it would be advisable to include the test of this virus in the selections programs (Komar et al., 2007; Cretazzo 2010). Even though no vectors of GFkV have been identified yet, this result leads us to suspect *in situ* spread of GFkV by some insect vector since this one is frequently transmitted simultaneously with GLRaV-3 (Cretazzo et al., 2010). Indeed, future epidemiological studies of GFkV in our collection would be required to improve our knowledge in this virus and its vector-borne transmission.

Under natural condition, GFLV is transmitted from grapevine to grapevine by the parasitic nematode *Xiphinema index* in a non-circulative manner (Andret-Link et al., 2004). The incidence of GFLV in this study is not surprising as it could be explained by the low presence of nematodes in the Germplasm Collection, due to the treatment control of this vector. The same GFLV incidence was recently observed in a germplasm collection of *Vitis vinifera* L. cv. Tempranillo from la Rioja (Northern Spain), although

its presence implied a higher deleterious effect in growth, yield and grape composition (Martínez et al., 2016). In addition to vector-borne transmission, the high incidence of all the viruses observed in the local cultivars could also be explained by vegetative multiplication of infected stocks, since in the Balearic Islands there are no grapevine nurseries and there is no certified planting material of minority cultivars in the market. In general terms, our results revealed that the most local cultivars were highly infected and with more than one virus. Multiple viruses' infection can result in a more substantially negative effect on fruit quality than single infection (Kovacs et al., 2001). Based in the poor sanitary status of local planting material as well as the low possibility to find healthy disease free-plant, the implementation of clean plant material using biotechnological sanitation techniques, is considered the main component to preserve and recover those cultivars. Indeed, our study has focused in the elimination of double and triple virus infection in two minority cultivars, which were demonstrated to have high agronomic and oenological interest (Escalona et al., 2009a, 2009b; García-Muñoz et al. 2012; Bota et al. 2013).

As mentioned in the introduction, different techniques have been applied to virus elimination in order to produce certified material, free of the most dangerous viral pathogens (Panattoni and Triolo 2013). These techniques (such as, *in vitro* and/or *in vivo* thermotherapy, meristem culture and chemotherapy) have shown differential success depending uponthe virus species (Maliogka et al., 2015).

Our results highlight the effectiveness of both treatments (ST and CT) to eliminate multiple infections in Gorgollassa cv.. Interestingly, the Gorgollassa cv. plantlets that remained infected after sanitation processes were found to be infected only by GLRaV-4-9, noting that the latter were not addressed by EU Directive 2005/43/EC rules on the marketing of material for the vegetative propagation of the vine. Hence, sanitation processes has successfully resulted in the elimination of the most spread virus in the collection (GLRaV-3) as well as GFLV. The high GFLV elimination efficiency observed in Gorgollassa cv. is also in line with that reached in "Bidaneh Sefid' and 'Shahroodi" cvs in Iran, combining meristem culture and thermotherapy (Salami et al., 2009). Similarly, several authors have succeeded to eliminate GFLV as well as viruses within the family *Closteroviridae*, by combining thermotherapy and in vitro culture of

shoot or meristem (Valero et al., 2003; Youssef et al., 2009; Maliogka et al., 2009; Skiada et al., 2009).

Argamussa cv. displayed more difficulty to in vitro culture, these results should be carefully considered in future studies in order to enhance regeneration and survival rate of this cultivar. In addition, the sanitation rate obtained from infected Argamussa cv. plants by using shoot tip culture was very low; with only one GLRaV-3+GFkV-free plant. Remarkably, the non-sanitized plants remained infected only with GFkV (66.66 %), thus may indicate the difficulty to eradicate this virus. Presently, Bota et al. (2014) showed that the combination of either high temperature during summer in the field or growth chamber thermotherapy treatment with shoot tip culture was effective for the elimination of GFkV in Manto Negro cv. (25 % and 20 %, respectively). Contrariwise, others studies revealed that GFkV elimination in grapevine is insensitive to heat treatment (Savino et al., 1985; Panattoni and Triolo 2010). However some researchers, managed to eliminate GFkV from a clone of Chardonnay using only shoot apex culture (Komar et al., 2007). It seems that the success of thermotherapy depends not only on the virus species involved but also on the specific interaction between the pathogen and the specific genotype (Maliogka et al., 2009). Indeed, much work needs to be done in the case of Argamussa cv. in term of knowing the sensitivity of GFkV elimination to heat (*in vivo*/ natural) treatment. Moreover, GLRaV-3 virus elimination resulted easier than GFkV, highlighting the effectiveness of this technique to eliminate GLRaV-3. Similarly, the elimination of GLRaV-1 was obtained with a high efficiency (87.5 %) by using meristem tip culture (1mm) (Youssef et al., 2009). Remarkably, our results also indicated that all the viruses presented in both cultivars could be eliminated even from big explants such as shoot tips (1-3 mm). The use of large plant tissue (> 1mm) might attributed to arrive at a compromise between virus elimination and plants regenerated and also to minimize the risk of somaclonal variation as reported for somatic embryogenesis method (Gambino et al., 2006, 2011) genetic or phenotypic changes induced by tissue culture.

CONCLUSION

The survey conducted in the germplasm collection of the Balearic Islands revealed that the local cultivars were highly infected with simple and mixed infection, being GLRaV-3 the most common. Even though, GFkV and GFLV were also present with considerable incidence. This situation may consolidate the necessity of the application of selection programs for recovering local cultivars and obtain plants suitable for authorization and certification. Indeed, our study has optimized two sanitation protocols for double and triple viruses' eradication. Our results revealed the successful elimination of the most common virus (GLRaV-3) as well as GFLV using the methods reported in the current study. The application of thermotherapy in combination with shoot tips culture seem to be valuable because this method is simple, rapid and may have the possibility to eradicate up to three viruses in grapevine. Remarkably, virus elimination using only the shoot tips culture was also effective to obtain free Argamussa and Gorgollassa plants.

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Supplementary Table S1: Incidence of virus in each cultivar (%). To simplify the interpretation of the results, GLRaV-1,-3 were grouped as single pathogen (GLRaVs), being both viruses associated to the same disease (Grapevine leafroll).

		Virus incidence		
Local cultivars	**Virus free**	**GLRaVs**	**GFKV**	**GFLV**
Argamussa	0	100	100	0
Batista Felanitx	0	100	20	0
Callet	0	100	22.22	0
Callet Negrella	0	71.42	42.85	0
Calop Blanc	0	100	100	0
Calop Negre	0	100	100	0
Calop Roig	0	100	100	0
Escursac	30.0	70	0	30
Esperó de Gall	0	100	100	88.8
Fernandella	37.5	5	25.0	0
Fogoneu	11.1	88.8	0	11.1
GAFARRÓ	14.2	85.7	0	0
Galmeter	0	100	100	100
Giró Ros	60.0	40.0	0	0
Gorgollassa	10.0	40.0	0	90.0
Jaumes	12.5	87.5	25.0	75.0
Magdalena	0	100	0	0
Malvasia de Banyalbufar	31.2	68.7	6.2	6.2
Mamella Vaca	0	100	0	0
Mances de Capdell	0	94.4	50	44.4
Mancés de Tibus	70	10	0	20
Mandó	0	37.5	100	0
Manto negro	0	88.8	44.4	0
Mateu	0	100	0	0
Molinera	0	100	0	0
Moll	0	100	66.6	0
Quigat	0	100	100	0
Sabater	0	100	22.2	88.8
Sinso	0	100	22	0
Valent Blanc	0	100	58.3	100
Valent Negre	0	100	100	77.7
Vinater Blanc	0	100	100	0
Vinater Tinto	0	76.4	58.8	52.9

Chapter 2

PHYSIOLOGICAL AND METABOLIC CHANGES IN GRAPEVINES UNDER COMBINED WATER STRESS AND VIRUS INFECTION

PHYSIOLOGICAL AND METABOLIC CHANGES IN GRAPEVINES UNDER COMBINED DROUGHT STRESS AND VIRUS INFECTION

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ABSTRACT

Worldwide, agriculture production continues to be constrained by the combination of abiotic and biotic stresses. In the Mediterranean region, grapevines usually deal with water stress during their summer growth season. At the same time, grapevines are hosts to a large number of viruses from which grapevine virus-3 (GLRaV-3) is one of the most widespread and causes considerable economic losses in many vineyards. However, information concerning grapevine responses to the combination of water stress and viral infection under field conditions is scarce. In this study, viral loads, physiology and metabolism were characterized in two Majorcan grapevine varieties during the summers of 2013 and 2014, and subjected to individual and combined stresses. As would be anticipated under water stress, net photosynthesis (A_N) and all growth parameters were significantly decreased in both varieties and metabolic changes were indicative of a mild water stress. Under well-watered conditions, virus infection significantly reduced A_N in both varieties in 2013, while reductions in growth parameters were only observed in one cultivar. Correlations between photosynthetic parameters, virus concentration and metabolite changes strongly suggest that diffusional rather than metabolic limitations underlie A_N behavior in both infected grapevine varieties under well-watered. Under combined stress, no additive effect was observed in A_N and growth. However, the combined stress triggered specific metabolic responses that could not be predicted from the sum of the individual stresses. Moreover, respiration and biomass under combined stress were significantly correlated with changes in the levels of respiratory substrates. Overall, these results denote a specific adjustment of respiratory metabolism that can explain the maintenance of leaf carbon balance and growth in grapevines under combined stress conditions.

Key words: Combined stress, Grapevine leaf-roll-associated virus-3 (GLRaV-3), photosynthesis, respiration, metabolite profiling, specific effect.

INTRODUCTION

In Mediterranean climate areas, grapevine is predicted to be a vulnerable crop in future climate change scenarios since its growth season coincides with the lowest water availability (Flexas et al., 2010; Shultz & Stoll, 2010; Hannah et al., 2013). Grapevine responses to water stress have been widely studied at the physiological and molecular levels over the last two decades (Medrano et al., 2002; Bota et al., 2001; Medrano et al., 2003; Chaves et al., 2007; Morison et al., 2008; Flexas et al., 2010; Carvalho et al., 2016). Depending on its intensity, water stress results in water loss, reduction in carbon assimilation, partial loss of canopy leaf area as well as plant carbon balance alteration due to an increase in the respiration to photosynthesis ratio (Chaves et al., 2003; Flexas et al., 1998, 2002; Chaves et al., 2007; Souza et al., 2003, 2005b; dos Santos et al., 2007; Escalona et al., 2012). Nevertheless, only few studies have investigated the genotypic variability in the metabolic response to water stress in grapevine (Cramer et al., 2007; Chaves et al., 2009; Hochberg et al., 2013).

The effect of water scarcity on grapevine is often combined with viral infections since those are present in all major grape-growing areas worldwide. *Leafroll virus-3* (GLRaV-3), a phloem-limited virus, is one of the most widespread viruses worldwide and provokes considerable economic losses (Martelli et al., 2012). In Majorcan viticulture, the incidence of multiple and single viral infections is very high and GLRaV-3 is the predominant virus in local varieties (Cretazzo et al., 2010), due to its relatively high replication efficiency (Velasco et al., 2014). The effect of viral infection on plant metabolism is still far from being understood, with effects appearing to be highly variable (Balachandran et al., 1997; Sampol et al., 2003; Petit et al., 2006; Christov et al., 2007; Komar et al., 2007; Basso et al., 2010; Barón et al., 2012). Several works demonstrated that GLRaV-3 causes a reduction in leaf photosynthesis and respiration, a disruption in transport and accumulation of assimilates and mineral nutrition and hormonal unbalances all of which in turn, have direct consequences on all aspects of growth and cropping (Mannini et al., 1996; Sampol et al., 2003; Moutinho-Pereira et al., 2012; Endeshaw et al., 2014; Montero et al., 2016a, b, c). Interestingly, Montero et al. (2016b) have recently revealed that the presence of GLRaV-3 did not affect total biomass increment in Giró Ros cv. despite its effect on carbon assimilation. In addition, the lower carbon assimilation in virus infected plants was compensated by an adjustment of carbon losses by respiration thus explaining the absence of virus effect

on carbon balance and biomass increment (Montero et al., 2016b), and pointing to adjustments in central carbon metabolism. In this respect, changes in the levels of relevant primary and secondary metabolites triggered by GLRaV-3 have recently been associated to changes in photosynthetic activity in Malvasia de Banyalbufar grapevine cultivar (Montero et al. 2016c). However, the co-occurrence of water stress and virus infection has started to be investigated only very recently in grapevines (Pantaleo et al., 2016; El Aou-ouad et al., 2016).

The simultaneous occurrence of two or more stresses is common in many agricultural areas. There is a growing evidence that some of the plant responses to stress combination are specific to stress-combination in question and cannot be predicted/explained by the responses to the individual stress applied (Atkinson and Urwin, 2012; Suzuki et al., 2014). Within the context of the climate change, the occurrence of combined drought and heat stresses are perhaps the most common environmental factors limiting plants growth and yield in agricultural areas worldwide (Suzuki el al., 2014). As such, the responses of plants to this stress combination have been the subject of considerable research effort (Awasthi et al., 2014; Obata et al 2015; Carvalho et al., 2016). Nevertheless, little is known about plant responses to simultaneous abiotic and biotic stresses, which are thought to cause negative impacts on biomass and crop yield, depending on the crop species, developmental stage, as well as intensity and duration of each stress (Ramegowda and Senthil-Kumar, 2015). In addition to abiotic pressures, plants have to face the threat of infection in their natural habitat and must defend themselves from the attack of different pathogens, including fungi, bacteria and viruses (Hammond-Kosack and Jones, 2000; Atkinson & Urwin, 2012). Heat and drought and their interaction with pathogens are one of the most studied stress combinations in plants (Rizhsky et al., 2004; Mittler, 2006; Prasch and Sonnewald, 2013; Ramegowad and Senthil-kumar, 2015; Pandey et al., 2015). The effect of combined stresses may trigger synergistic or antagonistic, physiological, metabolic and transcriptomic responses in plants. Previous studies have shown that the effect of stress combination on plant physiological traits become additive -i.e with more deleterious effects as compared to single stresses (Pandey et al., 2015; Jin et al., 2016). For example, recent studies have shown that combinations of heat, drought and virus infection cause a more detrimental effect on biomass, growth parameters and yield than the effects of individual stresses in Arabidopsis, barley and other crops (Prasad et al.,

2011; Vile et al., 2012; Rollins et al., 2013; Prasch and Sonnewald 2013). There is a running debate if the metabolite responses to simultaneous stresses can be predicted from the responses to the single stress treatments (Rizhsky et al., 2004; Rasmussen et al., 2013, Prasch and Sonnewald 2013). For instance, a high accumulation of proline and soluble sugars was observed when the plants were subjected to combined virus (Turnip mosaic virus, TuMV) and heat as well to multiple stresses (virus, drought and heat) (Prasch and Sonnewald 2013). Similarly, the combined effect of virus and drought stresses on *Nicotiana benthamiana* plants showed increased accumulation of osmoprotectants such as proline, glucose, fructose and sucrose (Xu et al., 2008). In addition, virus infected plants also showed lower transpiration rates due to partial stomatal closure resulting in better water retention in leaf tissues, providing evidences for pathogen-induced drought tolerance in that study (Xu et al., 2008).

Plant metabolomics have been used by many researchers since it represents a powerful tool by which study metabolic networks. In order to dissect and understand plant metabolic responses to combined stresses, several experiments have been conducted under environmentally controlled conditions. However, information about plant metabolic changes in crop species grown under field conditions is scarce (Obata et al., 2015). Interestingly in this study on maize, the combined effect of drought and heat on metabolite profiles was well predicted by the sum of the effects of the two individual stresses (Obata et al., 2015).

While the effect of two or more abiotic stresses combinations have been recently highlighted to be of high interest in grapevine research (Carvalho et al., 2016), the effects of the combination of drought and virus on grapevine are essentially unknown. The aims of the current study were: (i) to characterize the physiological and metabolic responses of two Majorcan grapevine cultivars with different susceptibility to virus infection, Malvasia de Banyalbufar and Giro-Ros, under well-watered and water stress conditions in combination with virus infection; (ii) to assess if the observed responses to the combined stress are predicted from the sum of single stresses; and (iii) to explore the relationships between metabolite changes and physiological traits to further understand the mechanisms regulating carbon balance and metabolism under virus infection and its combination with water stress.

MATERIAL AND METHODS

Plant material and treatments

Experiments were carried out at the experimental vineyard of the *Universitat de les Illes Balears* (Palma de Mallorca. Balearic Island, Spain) in two successive summers (2013 and 2014; from July 8th to August 13th). Both experiments were performed using two grapevine cultivars: Malvasia of Banyalbufar and Giro-Ros.

Plants were obtained by direct rooting of 0.2 m cuttings of dormant canes selected from mother plants growing under field conditions in a twelve-year-old experimental vineyard sited at IRFAP center (*Institut de Recerca i Formació Agrària i Pesquera. Conselleria d'Agricultura Medi Ambient i Territori*, Palma de Mallorca, Balearic Island, Spain). Cuttings were collected from asymptomatically infected vines (VI), and non-infected vines (NI). Rooting was induced by using indolbutyric acid (IBA. 2g L^{-1}) and plants were maintained in a greenhouse under controlled conditions with soil temperature 26-28ºC, air temperature 23 ± 0.1 ºC and air humidity maintained at about 80%. When cuttings displayed 4-5 expanded leaves, they were transplanted into pots and grown outdoors in 10 L pots filled with organic substrate and perlite mixture (5:1). They were irrigated daily from May until the start of the experiment and supplemented three times per week with 50% organic-mineral fertilizer NPK containing (%): organic N. 5; P_2O_5. 8; K_2O. 15; MgO. 2; organic C. 17.4. humic acid. 5; SO_3. 15; Fe. 1; Zn $2x10^{-3}$; Mn $1x10^{-2}$. A layer of perlite was added to the surface of each pot to avoid water evaporation from soil. Water stress was imposed by witholding irrigation. The level of water stress was defined by the leaf maximum daily stomatal conductance (g_s) according to Medrano et al. (2002). In both experiments, five plants per treatments of Non-infected (NI) and GLRaV-3 infected (VI) plants were subjected to two irrigation regimes: field capacity (g_s> 200 mmolH_2O m^{-2} s^{-1}) and moderate drought (50 <g_s <100 mmol H_2O m^{-2} s^{-1}). Once the desired g_s values were achieved (typically 4-5 days after water withholding) pots were weighed daily in the evening and watered to compensate the consumption to maintain the same level of drought for four weeks. Well-watered plants were maintained at field capacity irrigation regime throughout the experiments.

Virus detection and quantification

The presence or absence of GLRaV-3 was verified in mother plants by enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977) using commercial coating and conjugate antibody preparations (Bioreba AG, Reinach, Switzerland). The infection level was quantified by real-time PCR reactions in five different plants per treatment as previously described in El Aou-ouad et al. (2016).

Gas exchange and Chlorophyll fluorescence measurements

Instantaneous gas exchange measurements were carried out at the end of July using five fully expanded leaves per treatment in five different plants at mid-day using an open gas exchange system (Li-6400; Li-Cor. Inc., Lincoln. NE) equipped with a leaf chamber fluorometer (Li-6400-40; LI-COR Inc.). Measurements of net CO_2 assimilation (A_N), stomatal conductance (g_s), transpiration (E) and internal CO_2 concentration (C_i) were performed at saturating light (1500 µmol m^{-2} s^{-1}) achieved with the red LED lamp of the system with an additional 10% blue light to maximize stomatal opening. CO_2 concentration in the leaf chamber (C_a) was set at 400 µmol CO_2 mol^{-1}air in the cuvette and the relative humidity of the incoming air ranged between 40 and 60%. Block temperature was maintained at 30ºC, while water vapour pressure deficit (VPD) was not controlled.

Chlorophyll fluorescence measurements were done simultaneously to gas exchange using with the integrated fluorescence chamber head (Li-6400-40; LI-COR Inc.). From the fluorescence measurements, the actual quantum efficiency of the photosystem II (PSII)-driven electron transport (Φ_{PSII}) was determined according to Genty et al. (1989) as:

$$(\Phi_{PSII}) = (F_m' - F_S)/ F_m'$$

Where F_s is the steady-state fluorescence in the light (here PPFD 1500 µmol $m^{-2}s^{-1}$) and F'_m the maximum fluorescence obtained with a light-saturating pulse (~8000 µmol m^{-2} s^{-1}). $\mathbf{\Phi_{PSII}}$ represents the number of electrons transferred per photon absorbed by PSII. The rate of electron transport (J) can be calculated as:

$$J\ (\mu mole^{-}\ m^{-2}s^{-1}) = \Phi_{PSII} \times PPFD \times \alpha$$

(α a term that includes the product of leaf absorption and the partitioning of absorbed quanta between photosystems II and I). α was determined from the relationship between Φ_{PSII} and Φ_{CO2} obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing < 1% O_2 (Valentini et al., 1995).

Estimation mesophyll conductance by gas exchange and chlorophyll fluorescence

From combined gas-exchange and chlorophyll a fluorescence measurements, the mesophyll conductance for CO_2 (g_m) was estimated according to Harley et al. (1992)

$$g_m = A_N/(Ci - (\Gamma^*(J_{flu} + 8 (A_N + Rd)) / (J_{flu} - 4(A_N + Rd))))$$

Where A_N and C_i were taken from gas exchange measurements at saturating light. Γ* is a chloroplastic CO_2 photocompensation point and R_d is day respiration. Γ* was calculated according to Bernacchi et al. (2002) using the values of τ previously determined *in vitro* at 25°C in grapevines (Bota et al., 2002) and then recalculated at 30ºC according to Epron et al. (1995) and Valentini *et* al. (1995). In the experiments, night respiration (R_n) was used as a proxy for R_d by dividing R_n by 2 (Villar et al., 1995; Niinemets et al., 2005). R_n was determined by gas-exchange (Li-6400) measurements (n = 5) at 30ºC, after plants had been dark-adapted for more than an hour. Night respiration (R_n) was determined in a 6 cm^2 leaf chamber and air flow was set at 150 µmol s^{-1}.

Growth measurements

Whole plant leaf area and biomass were measured at the beginning and the end of the experiment. Total number of leaves per plant, shoot length and total leaf area were determined once a week during the experimental period. Leaf area (LA) was measured using an Image J program (Image J; Wayne Rasband/NIH. Bethesda. Maryland. USA) from direct images of all leaves of each plant at the onset of the experiment. During the experiment, LA was also estimated using mathematical relationship between leaf area and length (Villegas et al., 1981; Beerling and Fry, 1990). At the beginning and the end of the experiment, leaves, stems and roots from each plant were separated and dried in an oven at 60ºC to obtain the dry weight. Whole plant biomass was obtained from dry weights of roots, stems and leaves. Total plant biomass

increment was obtained from the difference in weight at the beginning and the end of the experiment.

Metabolite profiling

Metabolite analysis was performed in the same leaves used for gas-exchange measurements. Leaves without the midrib were sampled at midday and immediately frozen in liquid nitrogen for subsequent analysis. Leaf powder obtained by quick grinding under liquid nitrogen was extracted, derivatizated and subsequently analyzed by gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) as previously described (Lisec et al., 2006). The GC-TOF-MS system was composed of a CTC CombiPAL autosampler, an Agilent 6890N gas chromatograph and a LECO Pegasus III time-of-flight mass spectrometer running in EI+ mode. Metabolites were manually annotated by comparison with database entries of standards with aid of TagFinder software (Kopka et al., 2005; Schauer et al., 2005; Luedemann et al., 2008). The parameters used for peak annotation are shown as Supplementary Table 1.

Data and statistical analysis

Analysis of variance (ANOVA) was performed to reveal differences among cultivars, treatments and the interaction cultivar x treatment in the physiological and growth studied parameters. Significant differences between means were revealed by Duncan analyses ($P < 0.05$), performed with IBMSPSS statistics 16.0 (SPSS). Box plots of A_N, R_{leaves} and virus vine level were obtained using Sigma Plot 10.0 software package (Systat; Chicago, IL, USA).

To process the GC-MS results, intensity of a selected unique ion shown in Supplementary Table 1 was normalized to that of ribitol which is added to each sample as an internal standard as well as to the dry weight of the materials used for metabolite extraction. The value for each metabolite was divided by the mean of those from corresponding WW-NI samples and represented as “metabolite level” showing relative level of a metabolite compared to control condition (Supplemental Table 2).

Statistical analyses and graphical representations (ANOVA, Tukey’s HSD test, heatmap, hierarchical clustering, boxplots, Venn diagram, correlation analysis) were performed using the R-software environment 3.1.1 (http://cran.r-project. org/). The box plots were drawn by ggplot function in ggplot2 package. ANOVA and Tukey HSD test

was conducted by glht function in multcomp package. Correlation was tested by cor.test function and scatter plots were drawn by ggplot function. Venn diagram was drawn using venn.diagram function in VennDiagram package. Effects of the stress combination on metabolite levels were predicted from response in single stresses as follows. Response factors were calculated by $\log_2$ transformation of metabolite levels. Predicted response factors in DS+IV condition are the sum of those in DS and IV conditions. The values calculated from each cultivar in each year were considered as a replicate (n=4) and used for the calculation of the mean and SEM. Correlation between actual and predicted response factors were tested by cor. test function in R.

RESULTS

Absolute quantification of the virus

Under well-watered (WW) condition, virus concentration (GLRaV-3) was significantly ($P<0.05$) higher in Malvasia than Giro-Ros in the first experimental year (2013) (Fig. 1). However, no significant ($P<0.05$) differences in GLRaV-3 were observed between the cultivars in the second experimental year (2014). Under water stress (WS) conditions, the virus concentration was significantly ($P<0.05$) lower in Giro-Ros during 2014 as compared to Giro-Ros during 2013 and Malvasia in both years.

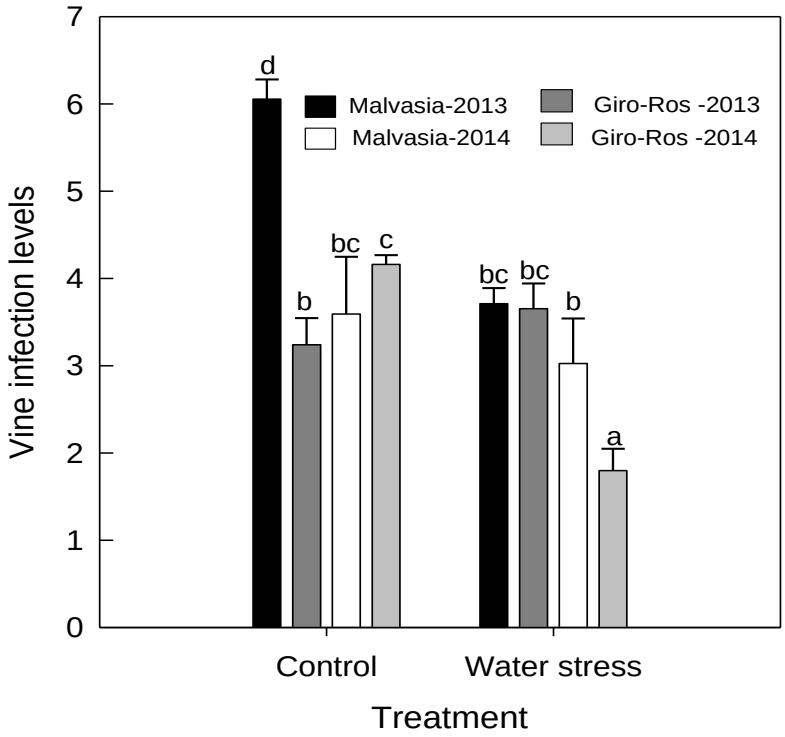

Figure 1: Mean log values (number of genome copies mg^{-1} host tissue) and standard deviations (SD) of the absolute quantities of GLRaV-3 of two cvs. Malvasia de Banyalbufar and Giro-Ros under control and water stress condition. Mean population of the virus was expressed as log_{10} of the viral copy numbers per 70 mg of leaves, collected from middle sections of grapevine shoots, used in the RNA extractions.

Effects of virus infection, water stress and combined stress conditions on growth parameters

As perhaps would be anticipated, the imposition of water stress induced a reduction in leaf area, shoot length, leaves per plant as well as total biomass increment (TBI) and the fractional biomass of different parts of plants (Table 1 and 2) in both cultivars and both harvests. Malvasia de Banyalbufar was the more affected cultivar by WS (Table 1). Generally, the presence of virus did not significantly affect plant growth parameters in either cultivar and year (Tables 1 and 2). However in 2013 Malvasia de Banyalbufar displayed a reduced total leaf area, leaf area increment, shoot length and TBI following infection. Under WS, virus infection (WS-VI) did not provoke a further detrimental effect on growth parameters i.e.- as compared to WS-NI, with the exception of Giró Ros plants in 2013 which displayed a lower leaf number per plant.

Table 1: Leaf area (LA), Increment leaf area, Shoot length and Total number of leaves (leaves per plants) measured in non-infected (NI) and virus-infected (VI) plants under well watered (WW) and water stress (WS) conditions. Values are mean of four-five replicates ± standard errors. Letters denote significant differences between treatments in each cultivar and experimental year ($P < 0.05$) according to Duncan's test.

	Treatments	Leaf area (m^2)	Increment Leaf area (m^2)	Shoot length (m)	Leaves per plant
2013 Malvasia De Banyalbufar	**WW-NI**	0.27 ± 0.03^{b}	0.16 ± 0.02^{c}	4.12 ± 0.03^{c}	59.00 ± 2.22^{b}
	WW-VI	0.21 ± 0.02^{a}	0.11 ± 0.01^{b}	3.91 ± 0.04^{b}	63.00 ± 2.39^{b}
	WS-NI	0.18 ± 0.08^{a}	0.03 ± 0.00^{a}	1.98 ± 0.01^{a}	42.75 ± 1.09^{a}
	WS-VI	0.21 ± 0.05^{a}	0.02 ± 0.03^{a}	2.49 ± 0.017^{a}	48.04 ± 1.53^{a}
Giró Ros	**WW-NI**	0.36 ± 0.00^{bc}	0.18 ± 0.01^{b}	2.26 ± 0.05^{b}	52.00 ± 1.22^{bc}
	WW-VI	0.38 ± 0.02^{c}	0.18 ± 0.02^{b}	2.05 ± 0.15^{b}	47.00 ± 2.19^{b}
	WS-NI	0.22 ± 0.01^{a}	0.05 ± 0.00^{a}	1.29 ± 0.05^{a}	52.50 ± 2.88^{c}
	WS-VI	0.31 ± 0.01^{b}	0.08 ± 0.01^{a}	1.45 ± 0.09^{a}	36.75 ± 1.49^{a}
2014 Malvasia De Banyalbufar	**WW-NI**	0.21 ± 0.02^{bc}	0.05 ± 0.00^{ab}	3.52 ± 0.2^{e}	45.75 ± 2.95^{cd}
	WW-VI	0.17 ± 0.01^{ab}	0.07 ± 0.01^{b}	2.95 ± 0.31^{d}	40.25 ± 3.11^{bc}
	WS-NI	0.13 ± 0.00^{a}	0.01 ± 0.00^{a}	2.33 ± 0.21^{c}	29.66 ± 1.82^{a}
	WS-VI	0.12 ± 0.00^{a}	0.03 ± 0.00^{ab}	1.95 ± 0.17^{bc}	28.75 ± 2.01^{a}
Giró Ros	**WW-NI**	0.30 ± 0.04^{d}	0.13 ± 0.03^{c}	1.95 ± 0.20^{bc}	41.60 ± 4.18^{bc}
	WW-VI	0.40 ± 0.02^{e}	0.22 ± 0.01^{d}	2.39 ± 0.10^{c}	53.40 ± 1.66^{d}
	WS-NI	0.20 ± 0.01^{bc}	0.44 ± 0.01^{ab}	1.25 ± 0.07^{a}	35.75 ± 3.97^{ab}
	WS-VI	0.20 ± 0.00^{bc}	0.04 ± 0.00^{ab}	1.43 ± 0.00^{a}	36.00 ± 0.63^{ab}

Table 2: Total biomass increment (TBI), Leaf biomass/total biomass (Leaf/TB), Stem biomass/total biomass (Stem B/TB) and Root biomass/ total biomass (Root B/TB). Values are mean of four-five replicates ± standard errors. Letters denote significant differences between treatments in each cultivar and experimental year ($P < 0.05$) according to Duncan's test.

	Treatments	TBI (g)	Leaf B/TB (%)	Stem B/TB (%)	Root B/TB (%)
2013 Malvasia De Banyalbufar	**WW-NI**	117.8 ± 12.6^{d}	22.3 ± 0.8^{abc}	46.5 ± 0.7^{a}	31.0 ± 1.4^{a}
	WW-VI	95.5 ± 6.5^{c}	24.8 ± 2.6^{c}	43.8 ± 1.4^{a}	29.8 ± 2.6^{a}
	WS-NI	32.5 ± 3.4^{a}	19.1 ± 1.0^{ab}	48.5 ± 6.6^{a}	31.9 ± 5.5^{ab}
	WS-VI	33.8 ± 5.8^{a}	18.2 ± 1.8^{a}	47.8 ± 5.0^{a}	32.0 ± 3.5^{ab}
Giró Ros	**WW-NI**	97.9 ± 5.2^{cd}	23.9 ± 0.4^{bc}	36.9 ± 2.2^{a}	39.1 ± 2.2^{ab}
	WW-VI	85.2 ± 11.1^{c}	24.0 ± 1.7^{bc}	43.9 ± 1.7^{a}	31.9 ± 2.1^{ab}
	WS-NI	35.7 ± 3.7^{a}	20.0 ± 1.5^{abc}	39.2 ± 2.7^{a}	40.7 ± 2.3^{ab}
	WS-VI	57.7 ± 3.7^{b}	17.9 ± 0.8^{a}	38.6 ± 4.5^{a}	43.3 ± 4.8^{b}
2014 Malvasia De Banyalbufar	**WW-NI**	71.4 ± 9.9^{b}	28.6 ± 0.8^{d}	40.7 ± 3.7^{ab}	30.1 ± 1.7^{b}
	WW-VI	64.0 ± 9.2^{b}	28.7 ± 1.7^{d}	42.4 ± 1.9^{ab}	29.2 ± 3.4^{b}
	WS-NI	26.8 ± 6.8^{a}	22.4 ± 1.6^{bc}	47.6 ± 1.6^{bcd}	29.9 ± 0.8^{b}
	WS-VI	32.3 ± 4.4^{a}	24.6 ± 1.5^{bcd}	47.4 ± 2.1^{bcd}	27.8 ± 1.0^{b}
Giró Ros	**WW-NI**	133.0 ± 8.4^{c}	26.9 ± 2.8^{cd}	52.7 ± 3.7^{cd}	20.2 ± 1.1^{a}
	WW-VI	136.3 ± 5.0^{c}	26.2 ± 2.4^{cd}	54.1 ± 2.0^{d}	19.6 ± 1.6^{a}
	WS-NI	31.8 ± 3.1^{a}	16.3 ± 0.9^{a}	47.0 ± 2.8^{abc}	36.6 ± 1.9^{c}
	WS-VI	22.2 ± 1.6^{a}	19.8 ± 1.1^{ab}	39.7 ± 2.3^{a}	40.5 ± 1.5^{c}

Rates of photosynthesis and respiration under virus infection, water stress and combined stress conditions

The imposition of water stress (WS) to non-infected plants (NI) significantly decreased A_N in both cultivars and experimental years (Fig. 2). Under WW condition, virus infection resulted in a significant reduction of A_N in both cultivars in the 2013 but not in 2014 (Fig. 2). The combination of both stresses (WS-VI) also induced a significant decrease in A_N as compared to WW-NI in both cultivars. However, no significant differences were observed in A_N between plants under WS-NI and WS-VI (Fig. 2).

Changes in leaf dark respiration induced by virus infection were generally less pronounced than those observed in A_N under both water treatments, and differ between genotypes (Fig. 2). In 2013, R_{leaves} was significantly decreased under WS-NI in Giró Ros but not in Malvasia de Banyalbufar. In 2014, R_{leaves} significantly increased in Giró Ros and decreased in Malvasia de Banyalbufar under WS-NI as compared to WW-NI. Under WW condition, virus infection did not significantly affect R_{leaves} in both cultivars and experimental years. Under WS, virus infection only significantly affected (decrease) R_{leaves} in Giró Ros at 2013 as compared to WW-NI.

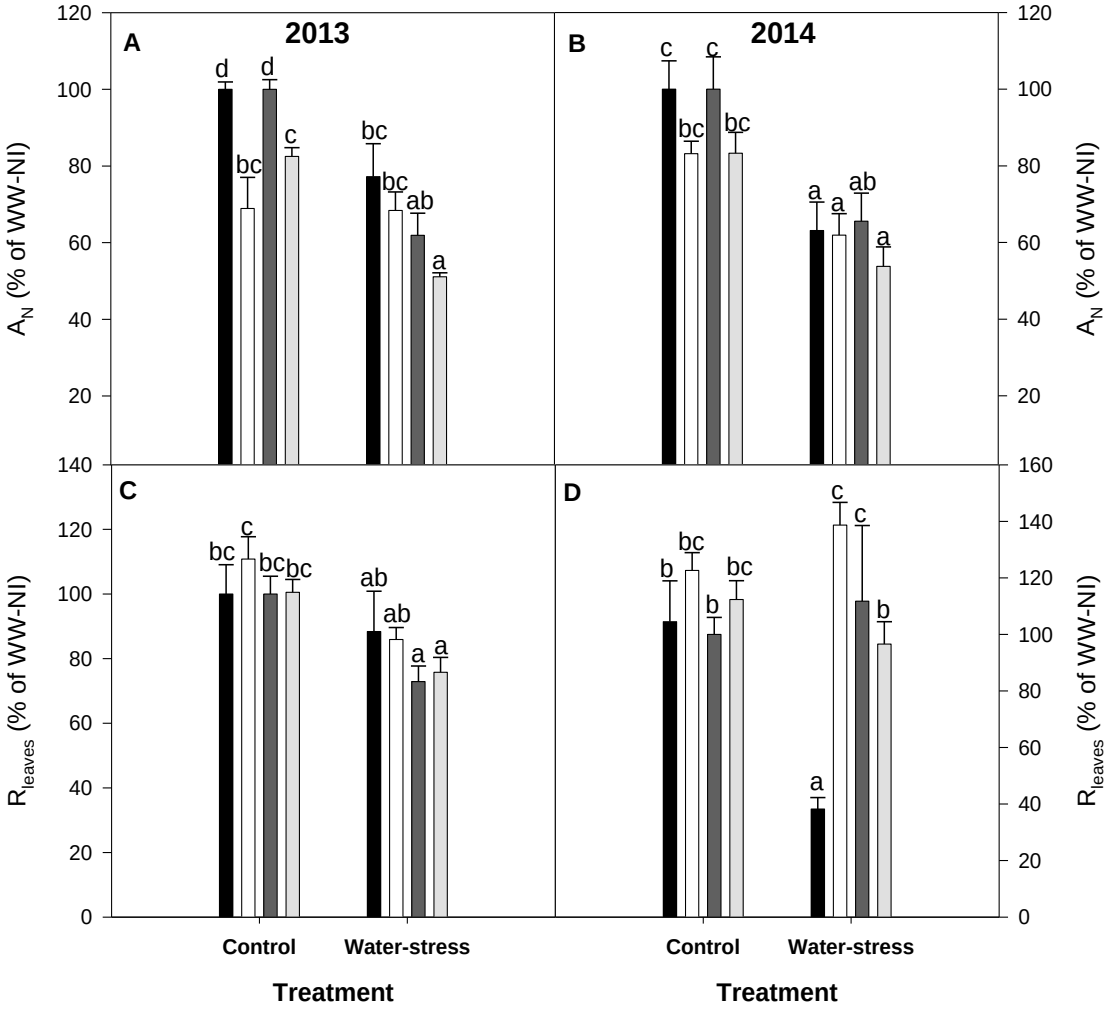

Figure 2: Net photosynthetic rate (A_N) and respiration rate at leaf level (R_{leaves}) in the two cvs. Malvasia de Banyalbufar and Giro Rós under control and water stress conditions. Values are mean of five replicates ± standard errors. Letters denote statistic significant differences by Duncan's test among treatments and cultivars in each experimental year ($P < 0.05$). Colours represent: Malvasia de Banyalbufar- NI (Black); Malvasia de Banyalbufar- VI (White); Giro Rós - NI (dark grey); Giro Rós- VI (light grey).

Relationships between virus concentration and gas exchange parameters

In order to understand the relationship between the virus concentration and the physiological traits determining carbon balance, we used all the data available to perform a correlation analysis between virus concentration (Log GLRaV-3 copy number), A_N, diffusive limitations (g_s and g_m) and R_{leaves} (Fig. 3). Under WW condition, virus concentration displayed significantly negative correlations with A_N, g_s, g_m (Fig. 3), but not with R_{leaves}. Contrariwise, a significant negative correlation was found only between virus concentration and R_{leaves} under WS.

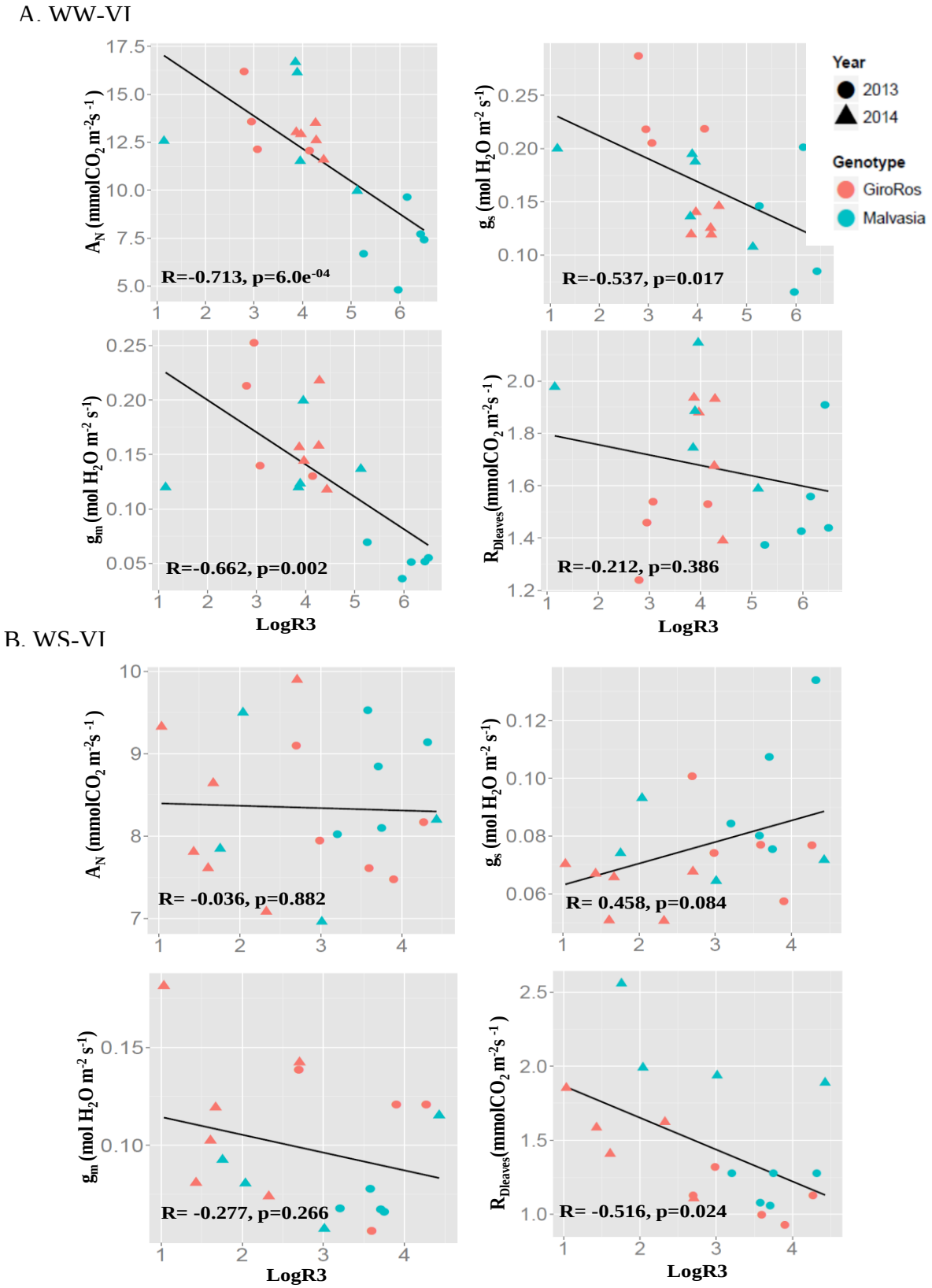

Figure 3: The relationships between GLRaV-3 concentrations (Log R3), net photosynthetic rates (A_N), stomatal conductance (g_s) and mesophyll conductance (g_m) under well watered (A. WW-VI) and water stress conditions (B. WS-VI). Circles and triangles indicate data from 2013 (n=10) and 2014 (n=10), respectively. Pearson correlation coefficients (R) and their corresponding P-values (P) are indicated.

Metabolite profiling under virus infection, water stress and combined stress conditions

The metabolic changes in both cultivars caused by water stress and virus infection were investigated using GC-MS based metabolite profiling. A total of 49 metabolites including 11 amino acids, 14 organic acids, 19 sugars and sugars alcohols and 5 other metabolites were detected in leaves of both cultivars, years and in all treatments (Fig. 4, Supplementary table 2). While a high variability was observed among cultivars and years, some common metabolic responses to the different stresses were identified in both cultivars (Fig. 4). Metabolites were separated into three different clusters by hierarchical clustering analysis (Fig. 4). The metabolites included in the first cluster (i.e. glucose, fructose, malate, threonate and 2-oxo-glutarate) tended to decrease in response to virus infection under well-watered (WW-VI) and water stress (WS-VI) conditions as compared to those in non-infected plants under well-watered (WW-NI) and water stress (WS-NI) conditions. Metabolites of the second narrow cluster, which included mainly amino acids (proline, glycine, glutamine and threonine) and phosphoric acid, tended to increase moderately under WS-NI and severely under the combined stress (WS-VI). Finally, in the third large cluster, the metabolite responses were similar under individual and combined stress conditions.

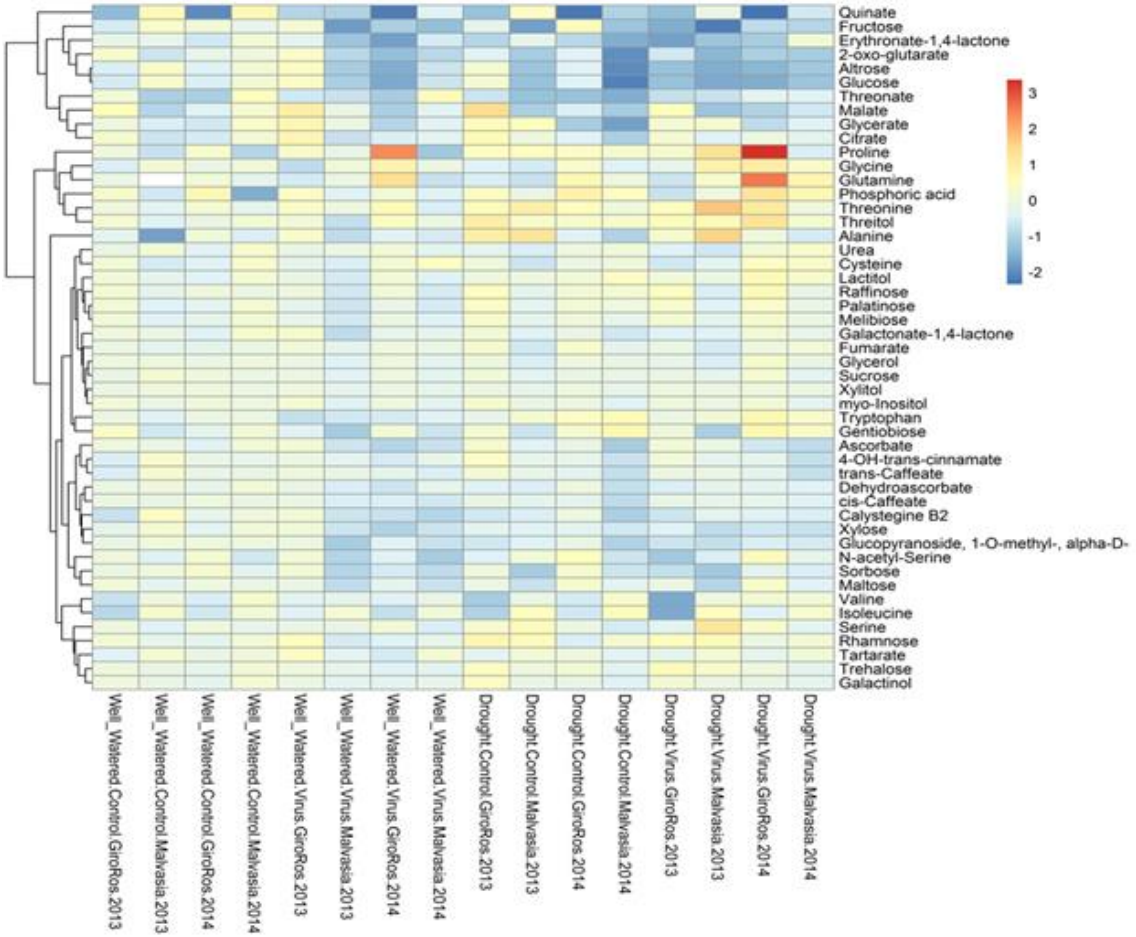

Figure 4: Heat map with hierarchical cluster analysis of metabolic responses to stress conditions in the two grapevine cultivars Malvasia de Banyalbufar and Giró Ros in both experimental years (2013-2014) under well watered (WW) and water stress (drought) conditions and combined or not with the virus infection. The value for each metabolite was divided by the mean of those from corresponding WW-NI samples and log2 transformed. Mean values after this normalization and transformation of six replicates are presented. Red and blue colors represent increase and decrease of metabolites using a false-color scale, respectively.

In order to highlight the conserved and consistent stress responses detected, we selected only the metabolites showing statistically significant changes under stress treatments with a similar trend in both years. Among the 49 metabolites detected (Supplemental Table S2), eight namely proline, threonine, glucose, fructose, sorbose, threitol, xylose, and 1-O-methyl-alpha-d-glucopyranoside fitted this criterion (Fig. 5). Among them, proline, threonine and threitol, displayed a significant accumulation specifically under stress combination (Fig. 5).

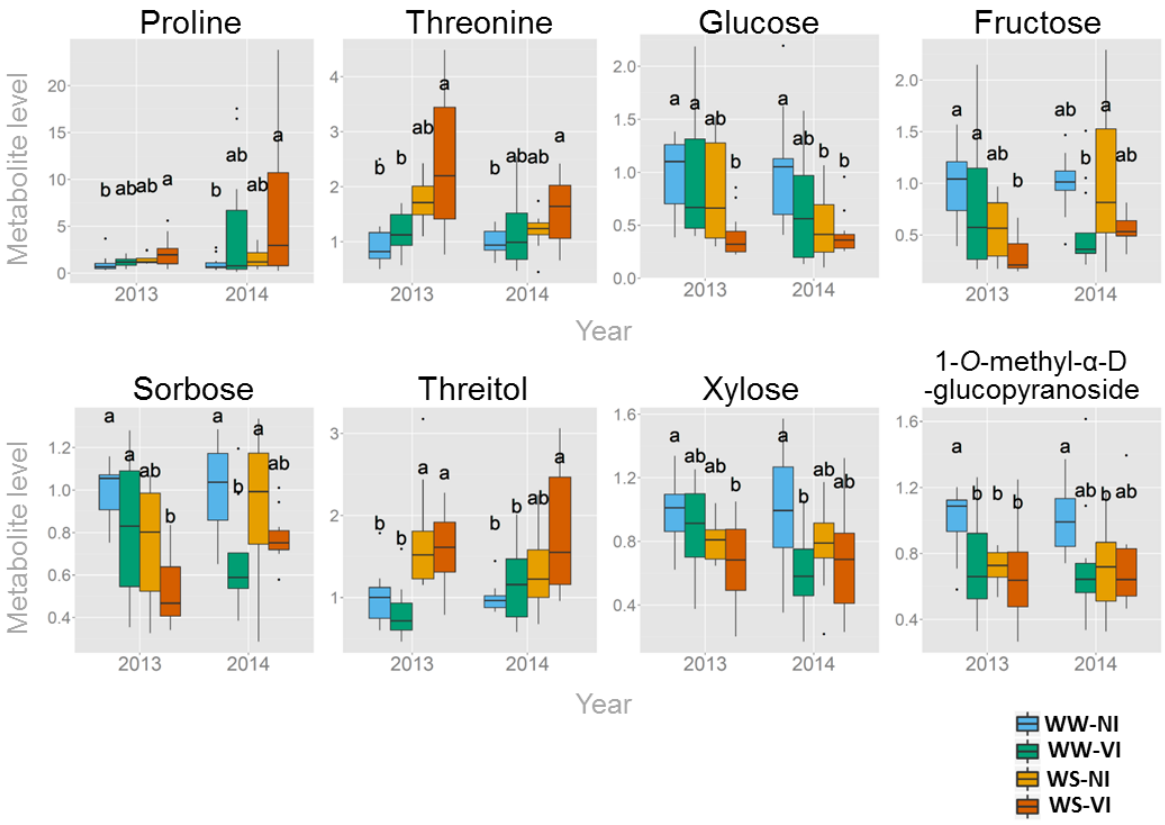

Figure 5. Box plots show the relative levels of selected foliar metabolites levels in both experimental years 2013 and 2014 combining the data of the two grapevine varieties. Only metabolites discussed in "Discussion section" are shown in this figure. Non-infected (control) and virus infected (virus) plants of both cultivars were grown in well-watered and drought conditions. Colors represents: WW-NI (blue), WW-VI (green), WS-NI (orange) and WS-VI (red) in two independent experimental years plots. Letters indicate the results of Tukey's test (P< 0.05) comparing the metabolites among treatments in each year (n=12).

Specificity of metabolic responses under combined stress

Among the metabolites significantly changed under combined stress (WS-VI) as compared to WW-NI, only two (1-O-methyl-alpha-d-glucopyranoside) and erythronate-1, 4-lactone (which both decreased) were shared with those changing under the individual stresses (Supplemental Fig. 1). Interestingly, this qualitative categorization shown in the Venn diagram revealed that seven metabolites (four increasing and three decreasing) specifically responded to the combined stress conditions (WS-VI) (Supplemental Fig. 1). All the metabolites specifically increasing under WS-VI were amino acids namely glutamine, glycine, proline and threonine, whilst those specifically decreasing included dehydroascorbate, glucose and fructose.

A more precise quantitative analysis comparing the sum of the response factors in WW-VI plus WS-NI with the actual response factor in WS-VI was performed in order to further analyze if the metabolic responses under combined stress are related to the ones of single stresses (Table 3). The response factor was calculated by dividing the metabolite level under stress conditions (WW-VI, WS-NI and WS-VI) by that in the WW-NI condition. Furthermore, the normalized data were $\log_2$ transformed and the correlation (r) between the sum of response factors in WS and VI (predicted response factor) and the actual response factor in the combined stress (WS-VI) were tested (Table 3). Remarkably, the predicted response factor was significantly correlated with the actual response for only nine of the 49 tested metabolites namely lactitol, melibiose, threonine, valine, malate and *trans*-cinnamate-4-OH, *trans*. caffeic acid calystegine B2, and quinate.

Table 3: Actual and predicted metabolites responses of two grapevine cultivars to well watered-virus infected (WW-VI), water stress-non infected (WS-NI) and (WS+VI) in both experimental years. Effects of the stress combination on metabolite levels were predicted from response in single (WW-VI) and (WS-NI) stresses as follows.

Metabolite	WW-VI[a]	WS-NI[a]	WS+VI[a]	Predicted[b]	r[c]
Alanine	0.20±0.35	0.67±0.88	0.73±0.92	0.87±1.21	0.942
Ascorbate	-0.42±0.21	-0.28±0.35	-0.22±0.30	-0.70±0.54	0.926
Calystegine.B2	-0.36±0.43	-0.40±0.28	-0.27±0.17	-0.76±0.68	**0.968**
cis.Caffeate	-0.28±0.11	-0.27±0.14	-0.16±0.05	-0.55±0.24	0.839
Citrate	0.02±0.18	-0.04±0.44	0.02±0.23	-0.02±0.60	0.812
Cysteine	0.10±0.19	-0.10±0.12	0.04±0.35	0.00±0.25	0.938
Dehydroascorbate	-0.27±0.08	-0.35±0.24	-0.30±0.11	-0.63±0.30	0.861
Erythronate.1.4.lactone	-0.77±0.17	-0.66±0.31	-0.85±0.34	-1.43±0.28	-0.7
Fructose	-0.78±0.59	-0.54±0.59	-1.25±0.41	-1.33±1.11	0.805
Fumarate	0.07±0.07	0.04±0.20	-0.03±0.11	0.11±0.27	0.643
Galactinol	-0.16±0.08	-0.05±0.20	-0.05±0.14	-0.21±0.27	0.925
Galactonate.1.4.lactone	-0.16±0.26	-0.20±0.23	-0.18±0.21	-0.36±0.46	0.702
Gentiobiose	-0.27±0.27	0.26±0.21	0.11±0.31	0.00±0.37	0.897
Glucopyranoside	-0.52±0.27	-0.52±0.17	-0.53±0.07	-1.04±0.37	0.568
Glucose	-0.46±0.49	-0.81±0.71	-1.27±0.27	-1.27±1.10	0.895
Glycerate	0.04±0.28	-0.28±0.66	0.05±0.34	-0.24±0.92	0.908
Glycerol	-0.20±0.10	-0.15±0.12	-0.12±0.17	-0.35±0.21	0.831
Glycine	0.26±0.37	-0.11±0.21	0.59±0.26	0.15±0.57	0.77
Isoleucine	0.01±0.07	0.11±0.20	-0.17±0.35	0.13±0.15	0.865
Lactitol	-0.08±0.25	0.22±0.13	0.33±0.22	0.14±0.34	**0.979**
Malate	0.17±0.35	-0.06±0.42	-0.38±0.20	0.11±0.72	**0.992**
Maltose	-0.37±0.12	-0.13±0.13	-0.19±0.20	-0.51±0.23	0.923
Melibiose	-0.20±0.19	0.07±0.11	0.11±0.14	-0.13±0.28	0.975
myo.Inositol	-0.10±0.11	-0.07±0.12	-0.03±0.08	-0.17±0.21	0.528
N.acetyl.Serine	-0.40±0.09	0.05±0.26	-0.19±0.39	-0.35±0.19	0.604
Palatinose	-0.27±0.20	0.13±0.10	0.11±0.18	-0.14±0.28	0.92
Phosphoric.acid	0.30±0.33	0.81±0.45	0.60±0.65	1.11±0.77	0.818
Proline	0.80±0.64	0.75±0.38	1.52±0.72	1.54±0.59	0.942
Quinate	-0.52±0.47	-0.55±0.41	-0.53±0.27	-1.07±0.76	**0.968**
Raffinose	-0.23±0.13	0.17±0.09	0.13±0.21	-0.07±0.16	0.869
Rhamnose	-0.05±0.15	0.33±0.23	0.37±0.19	0.28±0.29	0.652
Serine	0.05 ± 0.12	0.11±0.31	0.31±0.49	0.17±0.41	0.842
Sorbose	-0.47±0.24	-0.32±0.27	-0.64±0.21	-0.79±0.47	0.613
Sucrose	-0.18±0.13	-0.12±0.15	-0.11±0.14	-0.29±0.27	0.802
Tartarate	0.06±0.30	-0.06±0.11	0.04±0.03	0.00±0.32	0.448
Threitol	-0.02±0.24	0.56±0.12	0.74±0.31	0.55±0.26	0.724
Threonate	-0.07±0.31	-0.94±0.46	-0.20±0.45	-1.02±0.59	**0.91**
Threonine	0.22±0.15	0.54±0.24	0.92±0.42	0.76±0.32	**0.977**
trans.Caffeate	-0.10±0.18	-0.18±0.27	-0.21±0.19	-0.28±0.45	0.968
Trehalose	-0.12±0.09	0.13±0.17	0.26±0.19	0.01±0.24	0.926
Tryptophan	-0.44±0.18	0.35±0.25	0.33±0.22	-0.09±0.43	0.861
Urea	0.09±0.26	-0.11±0.18	-0.07±0.23	-0.03±0.38	0.685
Valine	-0.11±0.14	-0.22±0.25	-0.28±0.45	-0.33±0.28	**0.997**
2.oxo.glutarate	-0.43±0.19	-0.79±0.42	-0.84±0.06	-1.22±0.48	0.912
4.OH.trans.cinnamate	-0.16±0.18	-0.08±0.35	-0.18±0.28	-0.24±0.52	**0.963**
Xylitol	-0.13±0.08	-0.13±0.06	-0.06±0.04	-0.26±0.13	0.819
Xylose	-0.45±0.27	-0.32±0.15	-0.50±0.17	-0.77±0.39	0.892

[a]Response factors were calculated by $\log_2$ transformation of metabolite levels. [b]Predicted response factors in WS+IV condition are the sum of those in WS and IV conditions. Values calculated from each cultivar in each year were considered as a replicate (n=4). [c]Correlation coefficients between actual and predicted response factors. Values shown in boldface are significantly different from actual response factors in Student's t test ($P<0.05$).

Relationships between photosynthesis, respiration and metabolite levels under virus infection and its combination with water stress

From the results observed in Figure 3, it is clear that the effect of virus infection affected carbon balance (A_N and R_{leaves}) differentially depending of the presence or the absence of water stress. In order to better understand which metabolite responses are related to the effect of virus infection on carbon balance, correlations between metabolite levels and A_N or R_{leaves} were analyzed and compared between WW-VI and WS-VI conditions (Figure 6 and Supplemental Fig.2). Only cysteine and valine were significantly correlated to A_N under WW-VI and WS-VI conditions, respectively (Supplemental Fig S2). On the other hand, several metabolites significantly correlated with R_{leaves} in plants under both WW-VI conditions namely fructose, rhamnose, calystegine B2, citrate, ascorbate and malate and WS-VI conditions namely threonine, fumarate, erythronate 1-4-lactone, cysteine, rhamnose and trehalose (Fig. 6).

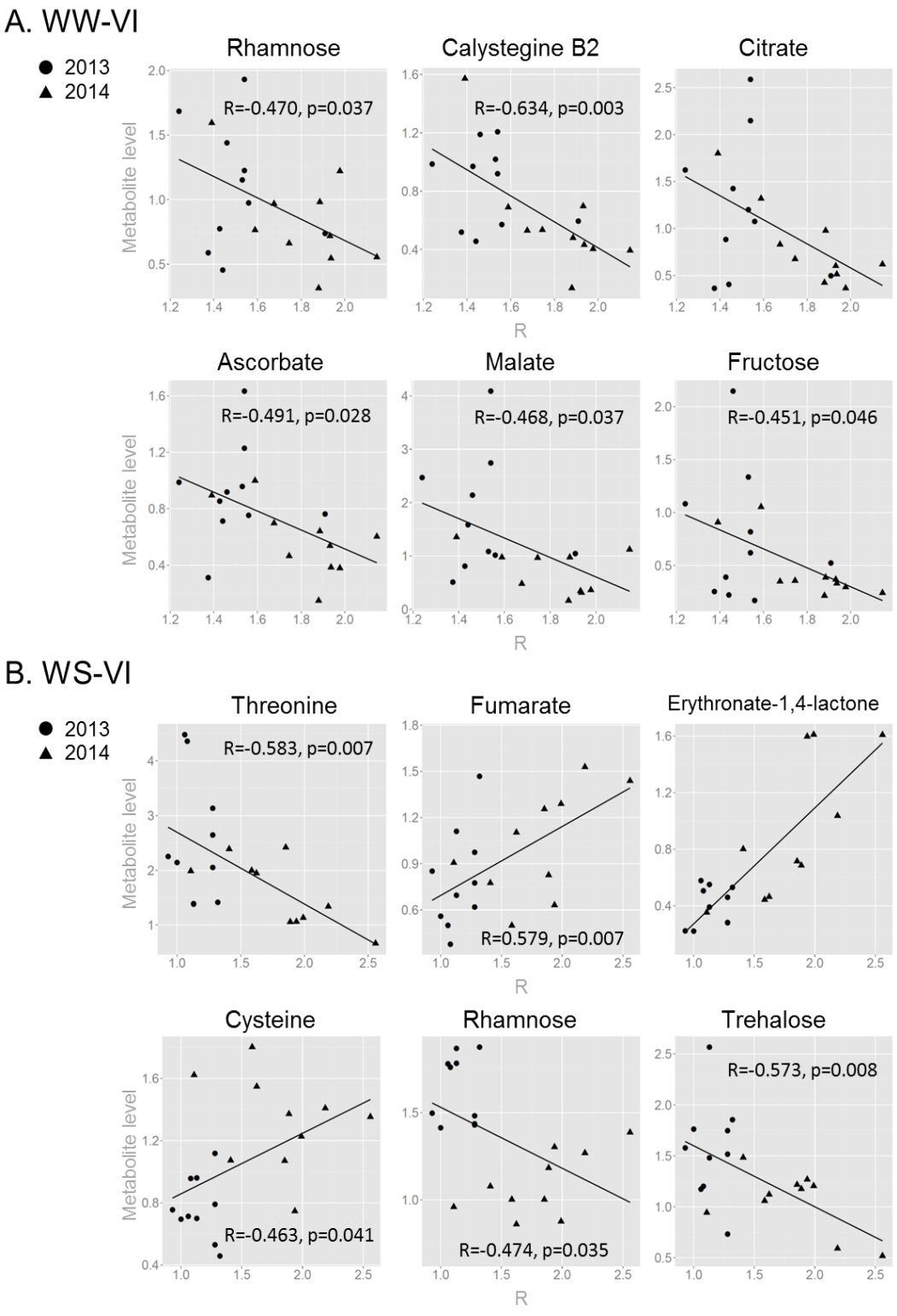

Figure 6: Relationship between leaf respiration (R, µmol CO_2 m^{-2} S^{-1}) and metabolites levels. Only the metabolites showing significant correlation ($p < 0.05$) with respiration are shown in this figure. Plots show correlations of 20 points corresponding to ten individual replicates in each year (2013, circles; and 2014, triangles) under well-watered (A-WW-VI) and water stress (B-WS-VI) conditions. Pearson correlation coefficients (R) and their corresponding P-values (P) are indicated.

Correlation between metabolites and plant biomass

As an integrative parameter of whole plant carbon balance, total biomass (TBI) was correlated with leaf metabolite levels under the different stress treatments applied (Table 4). The metabolites correlated with TBI were different in WW-VI, WS-NI and WS-VI. Under the WW-VI conditions, five metabolites were significantly correlated with TBI, being dehydroascorbate negatively correlated and raffinose, threitol, proline and threonine positively correlated to TBI. Under the WS-NI, only three metabolites (galactinol, myo-inositol and xylose) were positively correlated with TBI. However, twelve metabolites were significantly correlated with TBI under WS-VI condition. Among them, six amino acids (cysteine, glutamine, isoleucine, N-acetyl-serine and valine) showed strong negative correlation and three organic acids (ascorbate, citrate and malate) and three sugars (rhamnose, trehalose and 2-oxo-glutarate) were positively correlated with TBI (Table 4).

Table 4: Correlation between biomass increment and metabolites levels. Correlation coefficients are shown in each stress conditions, well-watered (WW-VI), water stress (WS-NI) and combined stress (WS-VI), using the data for both cultivars and years (n=12) in each condition.

Metabolite	WW-VI	WS-NI	WS-VI
Alanine	−0.10	0.33	0.02
Altrose	−0.43	0.34	0.27
Ascorbate	−0.32	0.30	0.58*
Calystegine.B2	−0.07	−0.02	0.05
cis.Caffeate	0.10	0.14	0.14
Citrate	−0.18	0.28	0.53*
Cysteine	−0.10	0.20	−0.69**
Dehydroascorbate	−0.61**	0.07	0.00
Erythronate.1.4.lactone	−0.37	0.25	−0.32
Fructose	−0.24	0.10	−0.29
Fumarate	−0.01	0.21	0.17
Galactinol	0.01	0.51*	0.41
Galactonate.1.4.lactone	−0.07	0.25	−0.15
Gentiobiose	0.33	0.04	−0.31
Glucopyranoside	0.02	0.01	0.12
Glucose	− 0.42	0.36	0.31
Glutamine	0.69*	0.02	− 0.69**
Glycerate	−0.28	0.27	0.26
Glycerol	0.42	0.10	−0.37
Glycine	0.43	−0.15	−0.35
Isoleucine	−0.39	−0.02	−0.58*
Lactitol	0.36	−0.13	−0.32
Malate	−0.27	0.33	0.73***
Maltose	0.22	0.06	−0.02
Melibiose	0.19	0.15	0.08
myo.Inositol	0.13	0.47*	0.16
N.acetyl.Serine	0.29	0.22	−0.57*
Palatinose	0.24	0.16	0.21
Phosphoric.acid	0.36	0.33	−0.73***
Proline	0.62**	0.22	−0.46
Quinate	− 0.40	−0.006	0.08
Raffinose	0.51*	0.33	0.01
Rhamnose	0.00	0.23	0.62**
Serine	0.28	0.38	−0.34
Sorbose	−0.21	0.30	−0.40
Sucrose	0.39	0.33	−0.21
Tartarate	−0.40	0.35	−0.26
Threitol	0.49*	0.40	−0.18
Threonate	−0.44	0.10	−0.05
Threonine	0.49*	0.31	−0.23
trans.Caffeate	0.09	0.11	0.35
Trehalose	−0.20	0.24	0.61**
Tryptophan	0.09	0.04	−0.47
Urea	0.41	0.19	−0.40
Valine	−0.20	−0.09	− 0.81***
2.oxo.glutarate	−0.38	0.05	0.54*
Trans.cinnamate.4.OH	−0.08	0.17	0.42
Xylitol	0.17	0.33	0.22
Xylose	−0.11	0.53**	0.16

*Correlation at P <0.05. **Correlation at P <0.001. ***Correlation at P <0.0001.

DISCUSSION

To our best knowledge, the results of this study highlight for the first time the interactive effect of virus infection and water stress conditions on the main physiological processes related to plant carbon balance and metabolite profiles in field grown grapevine.

Integrated effects of water stress on growth, carbon balance and metabolism

In this study, water stress clearly decreased plant growth in both cultivars. This was reflected in all relevant growth parameters, including as total leaf area, shoot length and total biomass accumulation (Table 1 and 2). Similarly, the effect of water stress on plant growth has been previously reported in many studies in grapevine (Schultz and Matthews, 1988; Poni et al., 1993; Escalona et al., 2002, 2003, 2012; Gomez-del-Campo et al., 2004; Van Leeuwen et al., 2009; Tomas et al., 2012). The limitation of plant growth imposed by water stress is mainly due to alteration in plant carbon balance, which is dependent on the balance between the intimately linked processes of photosynthesis and respiration (Flexas et al., 2010). Indeed, concomitantly with growth reduction, withholding water significantly decreased A_N in both cultivars (Fig. 2). The effect of water stress on photosynthetic capacity has been widely studied, including the analysis of diffusional and biochemical limitations of photosynthesis (Medrano et al., 2003; Tomas et al., 2013; Martorell et al., 2015a, 2015b; Bota et al., 2015; El Aou-ouad et al., 2016). By contrast, less is known about leaf respiration in response to water stress, with studies reporting increased, decreased or non-affected rates of water stressed plants (Flexas et al., 2005; Atkin and Macherel, 2009; Perdomo et al., 2014), including grapevines (de Souza et al., 2003; Gomez-del-Campo et al., 2004, Escalona et al., 2012; Zufferey 2016). Such variability on the response of leaf respiration was also observed in the present study (Fig. 2), and has been proposed to be related with different metabolic acclimations to water stress (Flexas et al., 2005; Galmés et al., 2007a). An accumulation of sugars including sucrose, glucose, and fructose, and amino acids including proline and threonine is involved in protective roles through several independent mechanisms (Rolland et al., 2006; Ramel et al., 2009; Krasensky and Jonak, 2012). On the other hand, increased amino acids and reduced sugars have been reported in grapevine cultivars (Hochberg et al. 2013) and also in others plants (Rizhsky et al., 2004; Prasch

and Sonnewald, 2013; Jin et al., 2016) subjected to a mild water stress. In agreement, mild water stress applied in this study (controlled by a stomatal conductance threshold) resulted in higher levels in proline and threonine (Figures 4 and 5) while soluble sugars such as glucose and fructose were not accumulated in the leaves of both cultivars and in both years (Figures 4 and 5), and even decreased in Malvasia de Banyalbufar.

Integrated effects of virus infection on growth, carbon balance and metabolism

Under well-watered conditions, reductions of photosynthesis have been commonly observed in several virus-plant interactions (Leon et al., 1996; Bertamini and Nedunchezhian, 2001; Hren et al., 2009), including virus infected grapevines (Margaria and Palmano, 2011; Baron et al. 2012; Margaria et al., 2013; Vitali et al., 2013; Kogovšek et al., 2015). In this respect, the accumulation of soluble sugars (sucrose, fructose, glucose) is thought to induce a metabolic feedback inhibition of photosynthesis in infected leaves, indicating a source-to-sink transition that further affects the mechanism of sugar transport and partitioning at the whole plant level (Bolton, 2009; Lemoine et al., 2009). In our study, the levels of soluble sugars (fructose, glucose) were not significantly changed or even were significantly decreased in some case (Fig. 4 and 5) under WW-VI, and therefore the observed significant reductions in photosynthesis (Fig. 2) cannot be explained by sugar accumulation (i.e. feedback inhibition) effects. Furthermore, very few significant correlations were found between A_N and primary metabolites (Fig. 6 and Supplemental Fig. 2). Following this vein, photosynthesis perturbation in the presence of virus was not explained by biochemical limitations but was mostly associated to diffusional limitations (g_s and g_m), since virus concentration was highly correlated with A_N, g_s and g_m (Fig. 3a, 3b and 3c), thus reinforcing previous observations suggesting that the virus induced decrease on A_N is associated to its effect on g_s and g_m (Endeshaw et al., 2014; El Aou-ouad et al., 2016). On the other hand, the results of the present study confirm that the VI do not significantly affect growth parameters neither total biomass increment in both cultivars (Montero et al., 2016b), with the exception of the case for Malvasia de Banyalbufar in 2013 in which the VI significantly ($P<0.05$) decreased different growth parameters (Table 1 and 2). We suggest that the observed growth and A_N differences in Malvasia de Banyalbufar between experimental years, as well as those observed between both cultivars, can be attributed to their different virus concentration. Indeed, the highest

virus concentration was detected in Malvasia de Banyalbufar 2013 plants as compared with those from 2014 and also with Giro-Ros (Fig. 1). A higher susceptibility to the *Grapevine leafroll associated virus- 3* (GLRaV-3) infection of Malvasia de Banyalbufar as compared with Giro-Ros was previously observed. Growth reductions have been recently reported in grapevine-virus interactions (Credi and Babini, 1997; Mannini et al., 1996; Sampol et al., 2003; Endeshaw et al., 2012; Endeshaw et al., 2014). The higher growth impairment previously observed as compared with the present study was probably due to a more pronounced effect of those viruses on leaf gas exchange parameters, including photosynthesis. Moreover, leaf carbon balance (A_N/R) was only significantly lower in VI plants displaying the highest (i.e. Malvasia de Banyalbufar 2013) virus concentration (data not shown). Leaf respiration rates in this study were not statistically affected, thus denoting the maintenance of an active respiratory metabolism during virus infection. Indeed, when data from both varieties was taken together, respiration rates under WW-VI were significantly correlated with several metabolites involved in respiratory pathways (Fig. 6A) including fructose and TCA cycle intermediates (citrate and malate). Previous works have reported a large effect of virus infection on respiration rates and the efficiency of the oxidative pentose phosphate pathway, glycolysis, and the TCA cycle (Técsi et al., 1996; Whitham et al., 2003; Espinoza et al., 2007; Babu et al., 2008). In summary, the observed responses in photosynthesis, respiration, levels of primary metabolites and their correlations suggest that alterations of the leaf carbon balance and growth in the presence of virus may not only relate to its effects on photosynthesis but are also driven by changes on respiratory metabolism (higher respiration rate linked to lower level of fructose and TCA cycle intermediates) which may be linked to the accumulations of plant defense compounds such as, some amino acids, antioxidant and cell-wall related metabolites (Berger et al., 2007; Gutha et al., 2010; Vega et al., 2011; Rojas et al., 2014; Montero et al., 2016c). Indeed, changes in amino acids (glutamine, threonine and proline), dehydroascorbate and raffinose induced by the presence of the virus under WW conditions were significantly and positively correlated to TBI (Table 4). Therefore, it appears that metabolic adjustments against virus infection in the current study are probably avoiding virus-induced growth reductions.

Integrated effects of the combined stress on growth, carbon balance and metabolism

Simultaneous drought stress and pathogen infection is one of the best studied stress combinations, triggering responses specific to the combination and also shared with individual stresses (Xu et al., 2008; Wang et al., 2009; Choi et al., 2013; Prasch and Sonnewald, 2013; Ramegowda and Senthil-Kumar 2015). The effect of combined stresses triggers synergistic or antagonistic responses in plants at different levels: physiological, metabolic and transcriptomic. Different studies have showed that the combination of abiotic stresses as well as the combination of biotic and abiotic stresses were generally additive and resulted in even more detrimental effect on growth traits as compared to the individual stresses (Prasad et al., 2011; Vile et al., 2012; Prasch and Sonnewald, 2013; Perdomo et al., 2014). By contrast, the results of the present study show that the combination of virus infection and water stress treatment did not further affect either plant growth or leaf gas exchange parameters, as compared to water stress alone (Tables 1 and 2 and Fig. 2). The absence of further effects on the combined stress could be explained by an effect of the water stress on suppressing viral replication. In this respect, virus concentration in Malvasia de Banyalbufar plants from 2013 and in Giró Ros plants from 2014 was significantly ($P<0.05$) lower under WS than under WW conditions (Fig. 1). A similar trend was observed in Malvasia plants from 2014. A beneficial effect of water stress over virus infection was previously reported by Xu et al (2008) in various plant species inoculated with four different RNA viruses, *Brome mosaic virus* (BMV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* and *Tobacco rattle virus* and by Pantaleo et al. (2016) in grapevine infected with *Grapevine rupestris stem pitting-associated virus* (GRSPaV). Nevertheless, a metabolic adjustment of plants under combined stress conditions that allowed maintenance of photosynthetic performance and growth cannot be discarded.

In support of a metabolic adjustment, our results showed that the combined stress caused more pronounced metabolic changes than virus or water stress individually (Fig. 5). Glucose, fructose and xylose were further decreased in the combined stress in most of the cases studied (Fig. 5) and are in agreement with previous observations in tomato plants infected with *B. cinerea* (Berger et al. 2004) and in sunflowers treated with *Sclerotinia sclerotiorum* (Jobic et al., 2007). In addition, an

accumulation of proline under combined stress was observed in both years, which is in line with the findings reported in CMV-infected beet plants (Xu et al., 2008) and in TuMV-infected Arabidopsis plants (Prasch and Sonnewald, 2013). Threonine was also increased under combined stress in our study, which, together with other amino acids such as isoleucine and methionine, were previously reported to increase in grapevines infected with Flavescence dorée, FDp (Prezelj et al., 2016) as well as after treatment with virulent pathogen (Ward et al., 2010). Threonine was previously identified as the amino acid involved in conferring resistance against some pathogens such as "oomycete *H. arabidopsidis*", by a dependent or independent of SA- and ROS-mediated defense pathways, presumably by altering the pathogen's ability to grow under that condition. In addition, the observed accumulation of threitol under combined stress in here (Fig. 5) can also be related with an osmoprotection mechanism as has been previously well described in endophyte infected plants under water stress condition (Dupont et al., 2015).

The significant and negative correlation ($P< 0.05$) found between virus concentration and R_{leaves} suggests that respiration can play a significant role on the metabolic adjustment under combined stress conditions (Fig. 3b). In close association to respiratory metabolism, fumarate and erythronate 1,4-lactone were found to be correlated to R_{leaves} under combined stress conditions which can be related with altered TCA cycle and ascorbate metabolism (Miura et al., 2004), respectively. Different lines of evidence suggest that TCA cycle (Nunes-Nesi et al., 2005) and the mitochondrial electron transport chain (Millar et al., 2003) are involved in ascorbate biosynthesis. On the other hand, R_{leaves} was significantly correlated with cysteine and threonine in our study, both related to the aspartate pathway-family amino acids. These amino acids are involved in energy production during stress via substrate supply to the TCA cycle and alternative respiratory pathways (Kirma et al., 2012). Additionally, our study revealed negative correlations between TBI branched-chain amino acids such as isoleucine and valine, which have also been proposed as alternative substrates feeding TCA cycle and respiration to obtain energy under stress (Araujo et al., 2011). Taken together, our results suggest an important role of amino acid metabolism under combined drought and virus infection perhaps for fueling mitochondrial respiration (i.e. higher respiration was associated with lower levels of threonine) to produce energy and carbon intermediates for the synthesis of defense compounds (i.e. ascorbate) as was previously suggested in other pathogen infections (Rojas et al., 2014; Prezelj et al., 2016).

Metabolic responses under combined stress are specific and related to respiration

Interestingly, the current work revealed that most of the metabolic changes under stress combination (WN-VI) were specific (Supplemental Figure S1) and not quantitatively predicted from the sum of responses to each single stress (Table 3), in contrast to what was observed for metabolite profile responses in Maize under the combination of drought and heat (Obata et al., 2015). Furthermore, the specificity of the metabolic response under combined stress is also supported by the correlation analysis results between metabolites and R_{leaves}; in such correlations, it is observed that most of the metabolites correlating with R_{leaves} under WW-VI (Fig. 6A) were different to those correlating with R_{leaves} under WS-VI (Fig. 6B), thus suggesting that respiration response to virus infection is linked to different metabolic adjustments occurring under single stress and under combined stress. Together with the metabolite changes and their relationships with respiration discussed in the previous section, our results reflect a metabolic adjustment that could prevent further physiological effects on plant carbon balance and growth under combined stress as compared to those effects under single stress conditions.

In summary, we have studied the physiological and metabolic responses of grapevines to *Grapevine leafroll-associated virus 3* (GLRaV-3) infection under well-watered and water stress conditions, and explored the relationships between leaf metabolites, carbon balance and plant growth under individual and combined stresses. Our results show that different physiological parameters such as photosynthesis and growth were affected under the different stresses, with Malvasia de Banyalbufar being more sensitive to the virus infection. However, no synergistic effect of the combined stresses was observed at the physiological level. At the metabolic level, responses to combined stress were specific and not quantitatively predicted from the sum of responses to each single stress. The observed alterations of primary metabolism in the presence of virus were closely linked to changes on respiratory metabolism. Therefore, the observed specific adjustments on (respiratory) metabolism can explain the maintenance of grapevines leaf carbon balance and growth under virus infection and its combination with water stress.

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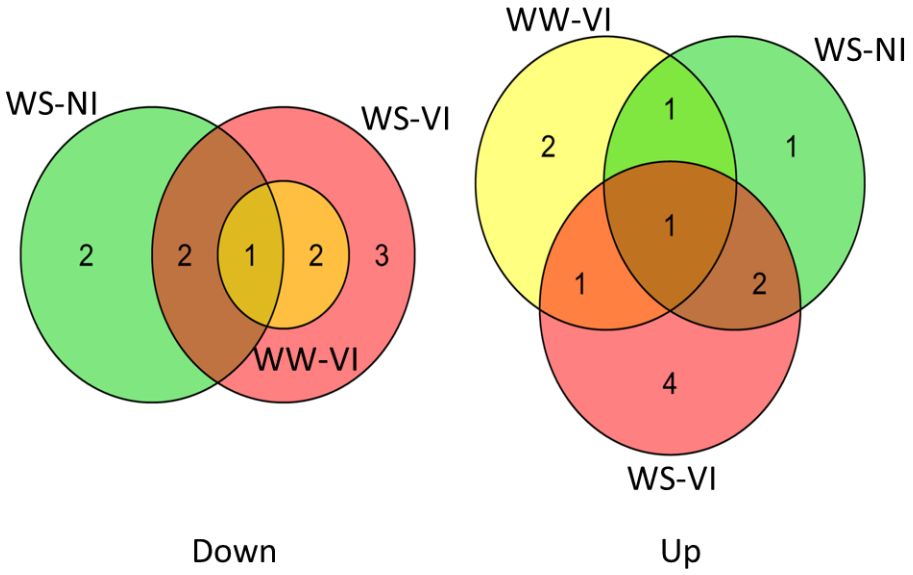
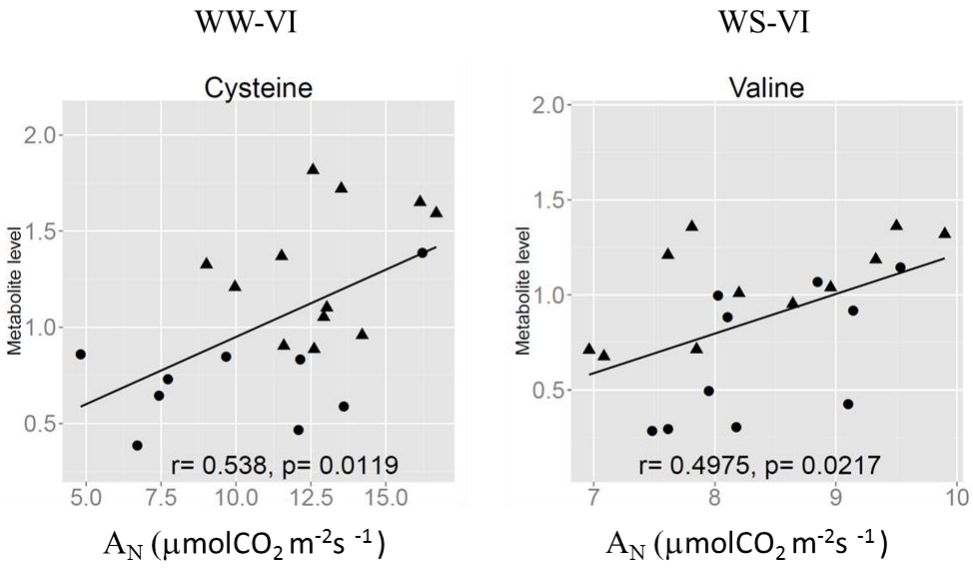


Supplemental Figure S1: Venn diagrams representing metabolic responses (down and up regulated) overlapping among individual (WW-VI and WS-NI) and combined stress (WS-VI). This diagrams revealed number of metabolites which altered levels in an individual and combined stress were significantly different from that in the well-watered conditions (WW-NI) by Tukey's test (p<0.05).



Supplemental Figure S2: Relationship between metabolites level and net photosynthesis rate (A_N) in response to virus infection under well watered (WW-VI) and water stress (WS-VI) conditions. Circles and triangles indicate data (n= 20) from 2013 and 2014, respectively. Pearson correlation coefficients (R) and their corresponding P-values (P) are indicated.

Chapter 3

INTERACTIVE EFFECTS OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3 (GLRAV-3) AND WATER STRESS ON THE PHYSIOLOGY OF *VITIS VINIFERA* L. CV. MALVASIA DE BANYALBUFAR AND GIRO ROS

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ABSTRACT

Among several biotic and abiotic stress combinations, interaction between drought and pathogen is one of the most studied combinations in some crops but still not in grapevine. In the present work, we focused on the interaction effects of biotic (GLRaV-3) and abiotic (drought) stresses on grapevine photosynthetic metabolism on two cultivars (cvs. 'Malvasia de Banyalbufar and Giró Ros). Non-infected and GLRaV-3 infected potted plants were compared under water stress conditions (WS) and well-watered (WW) conditions. Under WW condition, the results showed that photosynthesis (A_N) in both cultivars was decreased by the presence of GLRaV-3. The stomatal conductance (g_s) was the main factor for decreasing A_N in Malvasia de Banyalbufar, meanwhile reductions in Giró Ros were closely related to decreases in g_m. The observed differences in g_m between both cultivars might result from variation in their leaf anatomical, Giró Ros having higher values of g_m and leaf porosity (in all treatments). Moderate water deficit resulted in a closure of stomata and a decrease in g_m accompanied by a decrease in A_N in both cultivars. The maximum velocity of carboxylation (V_{cmax}) and electron transport rate (J_{max}) were also reduced under water stress. Moreover, the combined stress resulted in a reduction of most physiological parameters compared to healthy irrigated plants. However, no considerable differences were found between non-infected and virus infected (GLRaV-3) plants under water stress. Most of the results could be explained by the difference of virus concentration between cultivars and treatments.

Abbreviations: GLRaV-3, Grapevine leaf-roll-associated virus-3; WS, water stress; NI, non-infected; VI, virus infected

INTRODUCTION

In nature, plants are often simultaneously challenged by multiple biotic and abiotic stresses. However, plants able to tolerate a given stress occurring independently, may not be able to tolerate several stresses when that occur simultaneously (Atkinson and Urwin, 2012; Mittler, 2006, 2010; Nostar et al., 2013). Among several biotic and abiotic stress combinations, drought stress and pathogen is one of the most studied combinations in some crops but still not in grapevine (Carter et al., 2009; Ramegowda et al., 2013a; Sharma et al., 2007; Xu et al., 2008). Globally, water scarcity is the main limitation to agricultural production and this could be accentuated due to climatic change (IPCC 2013). In Mediterranean climate areas, grapevines usually deal with water deficit during growth period because most of its growth season copes with summer. This abiotic stress is often combined with viral infections since those are present in all major grape-growing areas worldwide. *Grapevine leafroll associated virus-3* (GLRaV-3) is one of the most widespread viruses worldwide and causes one of the most important diseases affecting grapevines comparable with several fungal diseases (Naidu et al., 2008; Martelli et al., 2012). In Majorcan viticulture, it's has been shown the incidence of multiple and single viral infections were very high and that GLRaV-3 was the predominant virus in local varieties (Cretazzo et al., 2010). Its high incidence in the field has been attributed to its higher replication efficiency than other grapevine leafroll viruses (Velasco et al., 2014). The water deficit problems are common in virus-infected grapevines thus both occur simultaneously; however, their effects on plants development are usually studied separately. Even though the responses of plants to simultaneous abiotic and biotic stresses are complicated (Garrett et al., 2006), the combined drought and diseases stresses have been reported in Xu et al. (2008) using Beet, pepper, watermelon, cucumber, tomato, zucchini, tobacco, *S. Habrochaites, C. amaranthicolor* and *N. benthamiana*. Their results indicate that virus infection improves plant tolerance to water stress and increased osmoprotectant and antioxidant levels in infected plants. Contrariwise, Pantaleo et al. (2016) has been reported that the combined water stress and *Grapevine rupestris stem pitting-associated virus* **(**GRSPaV)-infected plants influenced significantly photosynthesis rate, stomatal conductance and hydraulic resistance to water transport; via the effect of virus on miRNA expressions profiles of *V. vinifera* in response to drought. On the other hand, the research emphasis to understand the physiological and molecular bases of grapevine

responses to water deficits was deep undertaken in the last decade (Lawlor and Tezara, 2009; Vandeleur et al., 2009; Lovisolo et al., 2010; Chaves et al., 2010), but the grapevine responses to virus infection are still far from being well understood and they seems to be highly variable (González et al., 1997; Christov et al., 2001; Sampol et al., 2003; Petit et al., 2006; Komar et al., 2007; Christov et al., 2007; Basso et al., 2010; Montero et al., 2016a, 2016b, 2016c). Depending on the strain, GLRaV-3 can reduce yield, cluster size, delays fruit ripening and affects fruit composition and wine chemical profile of different *V. vinifera* cvs. like Cabernet Franc, Cabernet Sauvignon, Merlot (Borgo et al., 2003), Chardonnay (Komar et al., 2007) and Dolcetto (Mannini et al., 2012). Moreover, leafroll virus infections can negatively influence the resistance to biotic and abiotic stress and length of growing cycle and the vigour (Guidoni et al., 1997; Cabaleiro et al., 1999). Some works demonstrate that GLRaV-3 causes a drastic reduction in leaf photosynthesis during post-veraison (Gutha et al., 2012; Mannini et al., 2012). These effects are associated with other grapevine physiological disturbances like respiration, transport disruption and accumulation of assimilates, mineral nutrition and hormonal balance processes, which in turn have direct consequences on all aspects of growth and cropping (Mannini et al., 1996; Sampol et al., 2003; Moutinho-Pereira et al., 2012; Endeshaw et al., 2014). To our knowledge this is the first time that both stresses are studied in combination.

The aim of this study was to check the effects of GLRaV-3 infection and water deficit on leaf physiological parameters of Malvasia de Banyalbufar and Giró Ros, local grapevine cultivars of Majorca (Spain), identify which part of the photosynthesis machinery was mainly affected (diffusion or biochemical), and finally verify if one of the two cultivars has the same sensitivity to these combined stress.

MATERIAL AND METHODS

Plant Material and treatments

The experiment was carried out during summer 2012 and 2013 at the *Universitat de les Illes Balears* (Palma de Mallorca, Balearic Island, Spain) experimental field. In 2012 (from Jun 20th to July 30th), only Malvasía de Banyalbufar cv.was tested while in 2013 (from July 1st to July 31st) the experiment was expanded introducing Giró Ros cv.

Plants were obtained by direct rooting of 0.2 m cuttings of dormant canes selected from mother plants growing under field conditions in a twelve year old experimental vineyard sited at IRFAP center (*Institut de Recerca i Formació Agrària i Pesquera. Conselleria d'Agricultura Medi Ambient i Territori*, Palma de Mallorca, Balearic Island, Spain). Rooting was induced by indolbutyric acid (IBA, 2g L^{-1}) and plants were maintained in a greenhouse under controlled conditions. Soil temperature remained around 26-28ºC. Air temperature was 23±0.1 ºC and air humidity about 80%. When cuttings presented 4-5 expanded leaves, they were transplanted and grown outdoors in 10 L pots filled with organic substrate and perlite mixture (5:1). They were irrigated daily from May until the start of the experiment, supplemented three times per week, with 50% organic-mineral fertilizer NPK containing (%): N. 5; P_2O_5. 8; K_2O. 15; MgO. 2; organic C. 17.4. humic acid. 5; SO_3. 15; Fe. 1; Zn $2x10^{-3}$; Mn $1x10^{-2}$. The plants did not develop fruit.

A layer of perlite was added to the surface of each pot to decrease soil evaporation. Water stress treatment was defined by the leaf maximum daily stomatal conductance (g_s) according to Medrano et al. (2002). In both experiments, Non-infected (NI) and GLRaV-3 infected (VI) plants were subjected to two irrigation regimes: well-watered conditions (WW; g_s> 200 mol H_2O m^{-2} s^{-1}) and moderate water stress (WS; 50 <g_s <100 mol H_2O m^{-2} s^{-1}). Once stress values were achieved (typically 4-5 days after withholding water) pots were weighted daily in the evening and the amount of water consumed was replenished to maintain the same level of drought for four weeks. Control plants were maintained at field capacity throughout the experiments.
Soil water content (SWC) was calculated as:

$$SWC\ (\%) = \frac{(\text{pot weight} - \text{minimum pot weight})}{(\text{maximum pot weight} - \text{minimum pot weight})} \cdot 100$$

Minimum pot weight was considered to be at the wilting point. To determine wilting point, two pots per variety and treatment were left without irrigation. Pots were weighted until they achieved constant weight value at plant wilting. Maximum pot weight was obtained by weighting five pots per variety at field capacity (Fig.1).

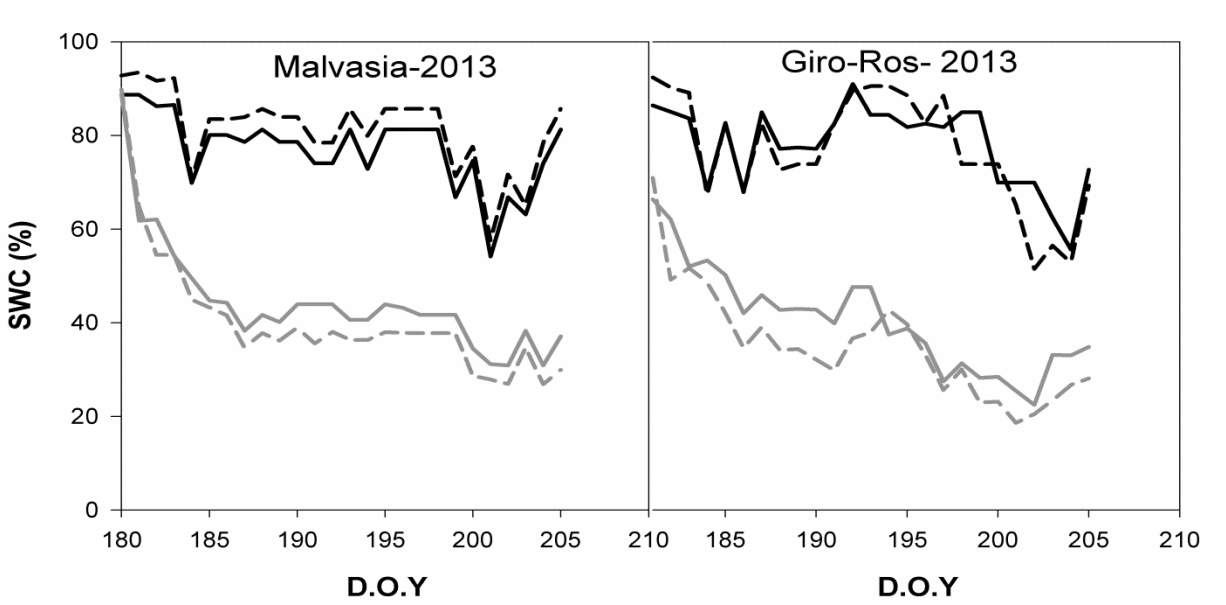

Figure 1: Soil water content (%) in Non-infected (NI) and GLRaV-3-infected (VI) Vitis vinifera plants under water stress and well-watered condition. Colour symbols are: black solid line (WW-NI), black short dish line (WW-VI), gray solid line (WS-NI) and gray short dish line (WS-VI), days of year (D.O.Y).

Plant water status and climatic conditions

Predawn leaf water potential (Ψ_{PD}) was measured in five fully expanded leaves per cultivar and treatment using Scholander pressure chamber (Soil moisture Equipment Corp. Santa Barbara. CA).

Climatic data (mean T (daily mean air temperature), max T (daily maximum temperature), min T (daily minimum temperature), evapotranspiration accumulated (ETPaccum), relative humidity and rainfall) were obtained using a weather station 7450 Groweather (DAVIS instruments Corp, Hayward. California. USA), located at the experimental field at the *Universitat de les Illes Balears* (Mallorca. Spain).

Virus detection and quantification

Firstly the presence of the following viruses: GLRaV-1, 2, 3, 4, 5, 7, 9, Grapevine Fanleaf Virus (GFLV), Arabis mosaic virus (ArMV) and Grapevine Fleck virus (GFkV) was tested in mother plants by enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977) using commercial coating and conjugate antibody preparations (Bioreba AG, Reinach, Switzerland). The plants qualified as non-infected and single GLRaV-3 infected by ELISA were selected for the experiment. Secondly, the ELISA results were confirmed by RT-PCR using a RT-PCR system (Illumina, SD,

USA) following the protocol of Pacifico et al. (2011). Total RNA from each sample was extracted (70 mg of phloem scraped from leaves) using Spectrum^{TM} Plant Total RNA Kit (Sigma-Aldrich, Inc.) according to the manufacturer's instructions. The Spectrum™ Plant total RNA Kit removes most of the DNA during RNA purification. However, for very sensitive applications, such as RT-qPCR, complete removal of traces of DNA may be necessary. On-Column DNase I Digest Set (Sigma-Aldrich Co., St Louis, MO, USA) was used to digest the DNA during RNA purification following the manufacturer´s instruction. RNA purity and concentration were measured at 260/280 nm using a spectrophotometer (NanoDrop-1000, Thermo Scientific, Villebon sur Yvette, France). First-strand cDNA synthesis with final volume of 20 µl was performed using 500 ng of total RNA, 200 units of recombinant Moloney Murine Leukemia Virus (MuLV) reverse transcriptase (Invitrogen Life Technologies, Inc.), 40 units of RNase inhibitor (RNase out, Invitrogen Life Technologies, Inc.), 0.4 mM of dNTPs, and 2 mM of random nonamers (Takara Bio, Inc.). The mixture for reverse transcription (20 µl) was incubated for 50 min at 37ºC and the reaction was inactivated by heating it at 70ºC for 15 min.

Quantification of the viral content was performed by real-time PCR reactions according to Montero et al. (2016c). Sequences of primers used in this study were retrieved from literature and used for amplifying partial gene-specific sequences. A list of primer pairs and amplicon lengths used for the virus diagnosis are provided in Table 1. GLRaV-3 standard curve for virus quantification was performed according to Pacifico et al. (2011) using RNA dependent RNA polymerase sequence (KP844580).

Table 1 Sequences of primers used for amplification of grapevine virus, amplicon length and references.

Virus	Primers	Sequences (5′-3′)	Size (bp)	References
GLRaV-1	GLRaV 1f	CAT CGC AAG ATG AGT CTG GG	275	Sefc et al. 2000
	GLRaV 1r	TTC ACA TTG CCC ACG CTG CC		
GLRaV-2	GLRaV-2 198 F	CATTATATTCTTCATGCCTCTCAGGAT	116	Osman et al. 2007
	GLRaV-2 290 R	GATGACAACTTCTGTCCGCTATAGC		
GLRaV-3	GLRaV-3 RdRp(1) For	TACGCTCATGGTGAAAGCAG	103	Montero et al.2016c
	GLRaV-3 RdRp(1) Rev	GGTTACGCACCTATCGTGGT		
GLRaV-4	HSP-85 F	ATATACATACCAACCGTTGTGGGTATAA	93	Osman et al. 2007
	HSP-178 R	CCCTATAAACTAGCACATCCTTCTCTAGT		
GLRaV-5	HSP-26 F	AACACTCTGCTTTTCTGCTGGC	162	Osman et al. 2007
	HSP-188 R	CTTTTTATGTCCCGATAAACGAGTACA		
GFkV	Fleck 239 f	CAACATCGAATGCCAATTTGG	89	Osman et al. 2007
	Fleck 328 r	GCCAGGCTGTAGTCGGTGTTGT		
GFLV	GFLV V1/F	ACCGGATTGACGTGGGTGAT	311	Osman et al. 2008
	GFLV C1/R	CCAAAGTTGGTTTCCCAAGA		

Modified from Pacifico et al. 2012

Gas exchange and Chlorophyll fluorescence measurements

Instantaneous gas exchange measurements were performed on 5 fully expanded leaves from middle shoot position from 5 different plants per treatment, using an open gas exchange system (Li-6400; Li-Cor. Inc., Lincoln. NE) equipped with a leaf chamber fluorometer (Li-6400-40; LI-COR Inc.). Measurements of net CO_2 assimilation (A_N), stomatal conductance (g_s), transpiration (E) and internal CO_2 concentration (C_i) were performed at saturating red light (1500 µmol m^{-2} s^{-1}) achieved with the red LED lamp of the system, with an additional 10% blue light to maximize stomatal opening. CO_2 concentration in the leaf chamber (C_a) was set at 400 µmol CO_2 mol^{-1}air in the cuvette and the relative humidity of the incoming air ranged between 40 and 60%. Block temperature was maintained at 30ºC within 1-2ºC variation and leaf-to-air vapor pressure deficit was maintained between 1 kPa and 2 kPa during all measurements.

The CO_2 response curves (A_N–Ci curves) were performed by varying the CO_2 concentration around leaves that have been previously acclimated to saturating light conditions (c. 15–20 min at a PPFD of 1500 µmol m^{-2} s^{-1}). The A_N–C_i curves were started at a C_a of 400 µmol CO_2 mol^{-1} air and then reduced stepwise (by 50 µmol CO_2 mol^{-1} air) until 0 µmol CO_2 mol^{-1} air was reached, providing at least 2 minutes at each step for stabilization. Thereafter CO_2 was increased stepwise from 400 to 2000 µmol CO_2 mol^{-1}air. Corrections for the leakage of CO_2 into and out of the leaf chamber of the Li-6400 have been applied to all gas-exchange data as described by Flexas et al. (2007).

Chlorophyll fluorescence measurements were done simultaneously to gas exchange using with the integrated fluorescence chamber head (Li-6400-40; LI-COR Inc.). From the fluorescence measurements, the actual quantum efficiency of the photosystem II (PSII)-driven electron transport (Φ_{PSII}) was determined according to Genty et al. (1989) as:

$$(\Phi_{PSII}) = (F_m' - F_S)/ F_m'$$

Where F_s is the steady-state fluorescence in the light (here PPFD 1500 µmol $m^{-2}s^{-1}$) and F'_m the maximum fluorescence obtained with a light-saturating pulse (~8000 µmol m^{-2} s^{-1}). $\mathbf{\Phi_{PSII}}$ represents the number of electrons transferred per photon absorbed by PSII. The rate of electron transport (J) can be calculated as:

$$J\ (\mu mole\ m^{-2}s^{-1}) = \Phi_{PSII} \times PPFD \times \alpha$$

(α a term that includes the product of leaf absorption and the partitioning of absorbed quanta between photosystems II and I). α was determined from the relationship between Φ_{PSII} and Φ_{CO2} obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing < 1% O_2 (Valentini et al., 1995).

Estimation mesophyll conductance by gas exchange and chlorophyll fluorescence

From combined gas-exchange and chlorophyll a fluorescence measurements, the mesophyll conductance for CO_2 (g_m) was estimated according to Harley et al. (1992)

$$g_m = A_N/(Ci - (\Gamma^*(J_{flu} + 8 (A_N + Rd)) / (J_{flu} - 4(A_N + Rd))))$$

Where A_N and C_i were taken from gas exchange measurements at saturating light. Γ* is a chloroplastic CO_2 photocompensation point and R_d is day respiration. Γ* was calculated according to Bernacchi et al. (2002) using the values of τ previously determined *in vitro* at 25°C in grapevines (Bota et al., 2002) and then recalculated at 30ºC according to Epron et al. (1995) and Valentini *et* al. (1995). In the experiments, night respiration (R_n) was used as a proxy for R_d by dividing R_n by 2 (Villar et al., 1995; Niinemets et al., 2005). R_n was determined by gas-exchange (Li-6400) measurements (n = 5) at 30ºC, after plants had been dark-adapted for more than an hour.

Calculated values of g_m were used to convert A_N–C_i curves into A_N–C_c curves according to the following equation: $\boldsymbol{Cc = Ci - (A_N / g_m)}$.

The biochemical reactions of photosynthesis are considered to be in two or sometimes three phases (according to von Caemmerer et al., 2000) obtained from the response of A_N to C_c. By fitting these phases key biochemical kinetic variables determining photosynthetic rate can be determined in vivo: the maximum velocity of carboxylation ($V_{c.max}$), the capacity for photosynthetic electron transport ($J._{max}$) and the triose-phosphate utilization rate (V_{TPU}). These model parameters on the basis of C_c are calculated using the temperature dependence of kinetic parameters of Rubisco described by Bernacchi et al. (2002).

Quantitative limitation analysis

To assess the limitations imposed by water stress, biotic stress (GLRaV-3) and the combination of both, a quantitative limitation analysis of photosynthesis was conducted for all three data sets according to Grassi and Magnani (2005). According to their approach measurements of A_N, g_s, g_m and $V_{c.max}$ were used to calculate the proportion of the three major components of total limitation for CO_2 assimilation: stomatal (SL) and mesophyll conductance (ML), as well as biochemical processes (BL). The maximum assimilation rate concomitantly with g_s and $V_{c.max}$ was reached under well-watered conditions; therefore the control treatment was used as a reference.

Total soluble protein and Rubisco Content

Leaf discs of 5.3 cm^2 from different plants, five replicates per treatment, were sampled and immediately frozen in liquid N_2. Samples were ground into powder in a mortar with liquid nitrogen. Extraction buffer was added and grinding continued until the mixture was thawed. The extraction buffer was composed of 100 mM N.N-bis (2-hydroxyethyl) glycine (Bicine)-NaOH (pH 8). 1 M dithiothreitol (DTT), 6% polyethylene glycol (PEG), 11 mM Na-diethyl-dithio-carbonate (DIECA), 1% (v:v) protease inhibitor cocktail (Sigma-Aldrich Co.. St.Louis. Mo.USA). Extracts were centrifuged at 14.500*g* at 4ºC for 2 minutes.

Leaf soluble protein concentration was determined spectrophotometrically according to Bradford (1976). Aliquots from different samples containing 3.5 μg of leaf soluble proteins were loaded in 12.5% SDS-polyacrylamide gel to determine the amount of Rubisco by quantitative electrophoresis by densitometry. Total proteins were separated by 0.75 mm thick of SDS-PAGE gel (12.5% resolving and 5% stacking). The gels were fixed in a mixture with water methanol and acetic acid for 1 hour stained in EZ Blue Gel Staining (Sigma-Aldrich Co. St. Louis. Mo.USA) solution for 1 hour and finally rinsed in water to remove stain excess. Gels were captured with a Chemidoc XRS densitometer (Bio-Rad) and images were analyzed by Quantity one 1-D (Bio-Rad) software.

Photosynthetic pigments

Chlorophyll *a*, *b* and total carotenoids were analyzed spectrophotometrically from the same crude extract used in leaf soluble protein analysis. Volume from different samples containing 50 μl of leaf extract were diluted in 950 μl of ethanol (96%) and after 10 minutes in dark, extracts were clarified by centrifugation (12000 rpm at 4ºC for 2 min.) and Chl a, Chl b and carotenoids were quantified using the equations according to Lichtenthaler and Wellburn (1983).

Leaf anatomy and Light microscopy

Small leaves pieces of 1x1 mm were cut between the main veins of three leaves per variety and treatment at the end of each experiment. Leaf material was quickly fixed under vacuum with 4% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). After primary fixation, the tissue was followed the detailed protocol as reported by Tomas et al., 2013.

Semi-thin cross sections (0.8 μm) were stained with 1% toluidine blue and viewed under Olympus light microscope BX60. Pictures were taken at 200x and 500x magnifications with a digital camera (U-TVO.5XC. Olympus) to measure whole leaf thickness and the thickness of epidermal layers, palisade and spongy mesophyll. Fraction of the mesophyll tissue occupied by intercellular air spaces (Mesophyll porosity) was calculated as:

$$f_{\text{ias}} = 1 - \frac{\sum S_{\text{s}}}{t_{\text{mes}} w}$$

Where ΣS_s is the total cross sectional area of mesophyll cells, w is the width of the section and t_{mes} is the mesophyll thickness between the two epidermises.

All images were analyzed with Image analysis software (Image J; Wayne Rasband/NIH. Bethesda. MD. USA) in 4-6 different fields of view, making ten measurements for spongy tissue and ten for palisade parenchyma cells for each anatomical trait.

Leaf mass per area and leaf density

Leaf area was measured using an Image J program (Image J; Wayne Rasband/NIH. Bethesda. Maryland. USA) from direct images of all leaves of each plant at the end of the experiment and then oven dried at 70ºC for 48 h and their dry mass was estimated. From these measurements, leaf dry mass per unit leaf area (M_A) was calculated. Using the estimates of leaf thickness from anatomical measurements, leaf density (D_L) was calculated as M_A per unit leaf thickness (Niinemets, 1999).

Statistical analysis

Regression coefficients, correlations and box plots were obtained using Sigma Plot 10.0 software package (Systat; Chicago, IL, USA). Analysis of variance (ANOVA) was performed to reveal differences among cultivars, treatments and the interaction cultivar X treatment in the studied parameters. Differences between means were revealed by Duncan analyses ($P < 0.05$), performed with IBM SPSS statistics 16.0 (SPSS).

RESULTS

Climatic conditions

Daily average weather data during the experimental period from May to August (in 2012 and 2013) are shown in Table.2. In general, the climatic variables registered in the two consecutive years were different according to mean, maximum and minimum temperatures. Significantly differences (±2°C) in mean air temperatures were showed, with the lowest being in 2013 (May and June) and the highest in 2012 (May and June). However, in July of 2013 the temperature was 2ºC higher than July 2012. Maximum temperatures were significantly different between two years, reaching more than 32°C in July of 2013. The minimum temperatures reached the highest values in May and June 2012. In contrast, in July and August, no differences were observed between two years. Rainfall and relative humidity were no significant during the experimental period, unless in May, humidity was different between both years. Accumulated evapotranspiration (ETPaccum) was similar for both years (Table 2).

Table 2. Climatic variables during the experimental period. The displayed values are: mean T (daily mean air temperature), max T (daily maximum temperature), min T (daily minimum temperature), evapotranspiration accumulated (ETPaccum) and rainfall per month Different letters denote statistically significant differences within each period at $P<0.05$ according to Duncan's test.

		Mean T (ºC)	Max T (ºC)	Min T (ºC)	RH (%)	ETPaccum (mm)	Rainfall (mm)
2012							
	May	$18.67^{aB} \pm 0.41$	$24.40^{aB} \pm 0.39$	$12.3^{aB} \pm 0.39$	$60.46^{abA} \pm 1.76$	132.69	12.1
	June	$23.96^{cD} \pm 0.37$	$30.07^{cdD} \pm 0.5$	$17.43^{cD} \pm 0.41$	$56.60^{aA} \pm 1.54$	138.50	0.20
	July	$24.89^{cdE} \pm 0.27$	$30.31^{cdD} \pm 0.39$	$18.81^{dE} \pm 0.38$	$60.17^{abA} \pm 1.68$	146.84	0.1
	August	$26.69^{eF} \pm 0.24$	$33.15^{eF} \pm 0.46$	$20.17^{eF} \pm 0.23$	$57.33^{eA} \pm 2.01$	100.39	0.1
2013							
	May	$16.55^{aA} \pm 0.3$	$21.31^{aA} \pm 0.48$	$11.04^{aA} \pm 0.33$	$65.26^{bB} \pm 1.28$	118.5	4.90
	June	$21.34^{bC} \pm 0.35$	$27.05^{bC} \pm 0.5$	$14.74^{bC} \pm 0.40$	$57.76^{aA} \pm 1.54$	146.5	2.40
	July	$26.10^{cEF} \pm 0.27$	$32.44^{cEF} \pm 0.4$	$19.31^{cEF} \pm 0.34$	$55.81^{aA} \pm 1.11$	168.2	0.60
	August	$25.65^{cEF} \pm 0.32$	$31.23^{cDE} \pm 0.54$	$19.69^{cEF} \pm 0.30$	$59.42^{aA} \pm 1.67$	128.4	4.5

Virus absolute quantification

The maximum virus concentration, 6.0 Log virus copy number, was observed in Malvasia under well-watered conditions. Contrariwise, in Giró Ros plants, the virus concentration was significantly lower than in Malvasia cv.. Under water stress treatment, the values obtained in both cultivars have shown the same magnitude as Giro-Ros in well-watered treatment ranging 3.2 to 3.7 Log virus copy number (Fig. 2).

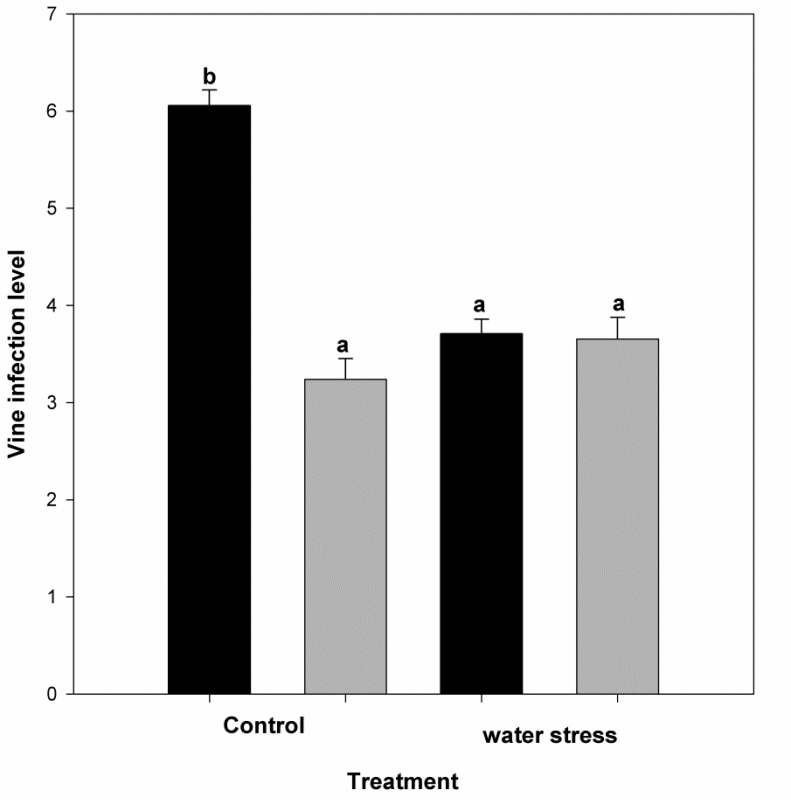

Figure 2: Mean log values (number of genome copies mg^{-1} host tissus) and standard deviations (SD) of the absolute quantities of GLRaV-3 of two cvs. Malvasia and Giro-Ros under control and water stress condition. Mean population of the virus was expressed as log_{10} of the viral copy numbers per 70 mg of leaves, collected from middle sections of grapevine shoots, used in the RNA extractions. Colour symbols are: black (Malvasia), gray (Giro-Ros).

Effects on gas exchange parameters

Under well-watered condition, a significant decrease in A_N and g_s were observed in Virus Infected (VI) plants in Malvasia cv., for both experimental years, while g_m was not affected by virus infection (Fig 3). Less pronounced A_N reduction was observed in Giró Ros.cv under well-watered conditions (Figs. 3A, B). However, this difference in A_N was accompanied by a decrease in g_m, while g_s remained unaffected (Figs. 3D, E). Under well-watered condition, g_m values were significantly different between both varieties. The highest values were observed in NI and VI Giró Ros cv. However, no differences were observed in g_m between both varieties under water stress. The imposition of water stress to non-infected plants induced a reduction in A_N, g_s and g_m, except in Malvasia cv. in 2013 where g_m seemed unaffected (Fig. 3D). No differences were observed in A_N, g_s and g_m, between NI and VI plants under water stress situation. However, the combined stress resulted in a mild reduction of all these parameters,

respect to control NI plants, except in Malvasia cv. in 2013. Another interesting point is that in Malvasia de Banyalbufar cv. (2012 and 2013), the results in VI plants in WW are similar to those measured VI plants in WS (Fig. 3).

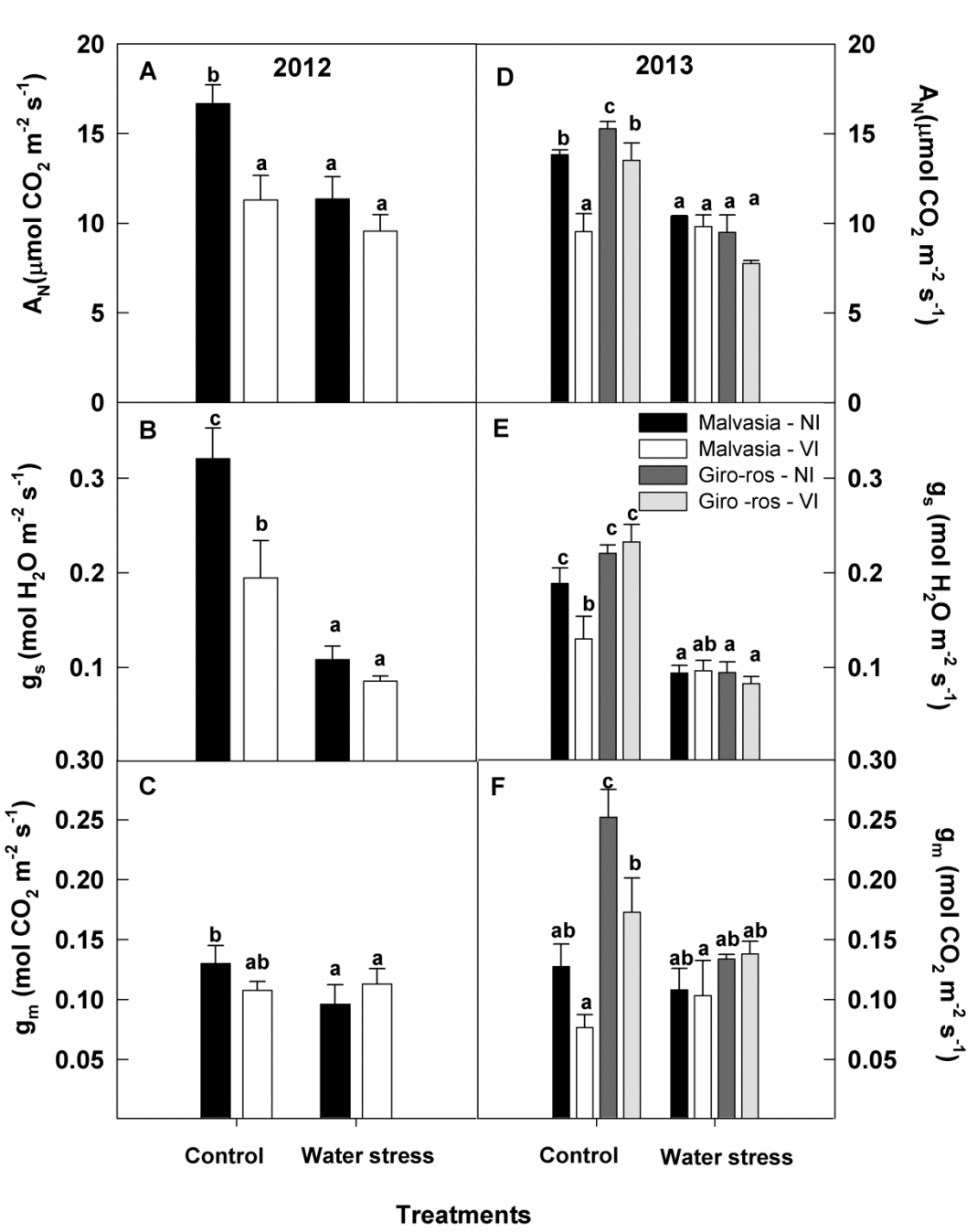

Figure 3. Variation of (A) net photosynthetic rates (AN). (B) stomatal conductance (gs) and (C) mesophyll conductance (gm) in Noninfected (NI) and virus-infected (VI) Vitis vinifera plants under water stress and well-watered condition in two consecutive years (2012 and 2013). Colour symbols are: black (Malvasia-NI), dark gray (Giro-Ros-NI). White (Malvasia-VI), light gray (Giro-Ros-VI). A_N and g_s values are means ± S.E six replicates while g_m values are means ± S.E five replicates. Different letters denote significant differences ($P < 0.05$) between treatments and cultivars (2013) and treatments in 2012 experiment.

In the first year of experiment (2012), $V_{c.max}$ and J_{max} were significantly higher in NI Malvasia.cv. plants under WW than in VI plants. Virus infection reduced $V_{c.max}$ and J_{max} by 53% and 34% respectively. During the second year of experiment, when both varieties were tested, the $V_{c.max}$ and J_{max} reductions were similar in %, but we found much more variability among plants, thus the differences were not significant (Table 3). The water stress supposed reductions in J_{max} in both years and both varieties. The plants subjected to both stresses presented the lowest $V_{c.max}$ and J_{max} values (Table 3).

Regarding limitation analysis, the total photosynthesis limitation by GLRaV-3 was higher in Malvasia (32%) than Giró Ros (12%). Partial limitations were similar in Malvasia, with SL (9%), ML (10%) and BL (12%) while in Giró Ros BL supposed 7% of the total.

Table 3. Changes in photosynthetic parameters in non-infected (NI) and virus-infected (VI) plants under control and water stress treatments. $V_{c.max}$, J_{max}, denote for maximum carboxylation rate, maximum photosynthetic electron transport rate in Malvasia and Giro-Ros. Values are means ± S.E five replicates. Small letters denote significant differences ($P < 0.05$) between treatments in each variety and capital letters denote significant differences ($P < 0.05$) according to Duncan's test between varieties (2013).

Treatment	**Control**		**Water-stress**	
	NI	**VI**	**NI**	**VI**
Malvasia-2012				
V_{cmax}	196.5^{c} ±12.0	143.5^{ab} ± 6.7	166.6^{bc} ±12.8	115.9^{a} ± 9.7
J_{max}	155.7^{b} ±10.3	121.8^{a} ± 5.9	115.1^{a} ± 14.3	97.5^{a} ± 7.4
Malvasia-2013				
V_{cmax}	210.7^{aA} ± 21.2	169.3^{aA} ± 25.4	207.8^{aA} ± 3.1	161.6^{aA} ± 16.4
J_{max}	151.0^{bC} ± 12.0	120.4^{abA} ± 15.3	129.6^{aABC} ± 3.5	102.5^{aAB} ± 5.8
Giro-ros-2013				
V_{cmax}	209.9^{bA} ± 24.9	151.3^{aA} ± 9.4	182.5^{abA} ± 6.7	152.9^{aA} ± 11.1
J_{max}	135.2^{bBC}±12.9	115.8^{abAB} ± 5.8	104.6^{aAB} ± 6.9	98.5^{aA} ± 6.1

Under water stress, the SL was the most important in both years and cultivars (Fig. 4). Both varieties showed a slightly increased in total limitation to photosynthesis under combined stress, as compared to control treatment (WW_{NI}-WW_{VI}). Tthe contribution of SL was the most important in both varieties and represent 60% of total limitation in case of Malvasia and 40% in case of Giró Ros (Fig 4).

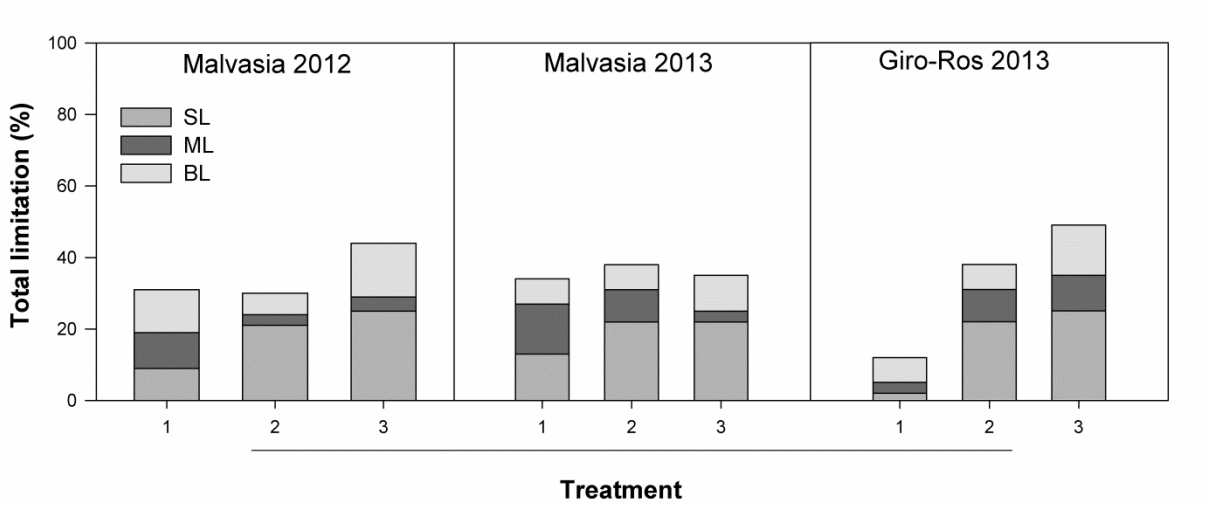

Figure 4. Quantitative Limitations analysis of photosynthesis, stomatal conductance limitation (SL), mesophyll conductance limitation (ML) and biochemistry limitation (BL), expressed as a percentage as compared to the control maximum values (%), calculated in three treatments: (1) GLRaV-3 effect in well-watered condition (WW_{NI}-WW_{VI}). (2) water stress effect in non-infected plants (WW_{NI}-WS_{NI}) and (3) combined stress effect (WW_{NI}-WS_{VI}).

Photosynthetic pigments and soluble proteins

The changes in photosynthetic pigments and soluble proteins in NI and VI plants under well-watered and water stress conditions were determined in 2013 and are shown in Table 4. The photosynthetic pigments and Rubisco content seemed unaffected by virus infection and/or water stress. Only an increase in Rubisco content was measured in water stressed NI Malvasia plants (Table 4).

Leaf anatomical components

Significant differences were observed in the different anatomical parameters of Malvasia leaves due to virus infection, while less pronounced differences were observed by water stress in NI plants. However, in Giró Ros both stresses suppose differences in the measured parameters (Table 5). Under WW, virus infection increased a 20 % leaves thickness (T_L) in Malvasia and 10% in Giró Ros. In response to water stress, leaf thickness increased only in Giró Ros (Table 5). In Malvasia, leaf density (D_L) was increased by VI. However, under WS D_L remained unaffected respect to well-watered condition. In Giró Ros D_L was not affected by VI under well-watered condition, but increased under combined stress (Table 5). Leaf mass area (M_A) and leaf density (D_L) were respectively 1.2 and 1.5 fold higher in Malvasia than Giró Ros.

Mesophyll porosity (f_{ias}) showed significant differences between both cultivars and treatments, ranging from 15 to 32%. Observed differences in Malvasia were not related to virus infection, but were attributed to water stress. However, in Giró Ros f_{ias} was decreased by virus infection and also by water stress. Moreover, mesophyll porosity was well correlated with mesophyll conductance in both cultivars under well-watered condition, but not under water stress (Fig. 5).

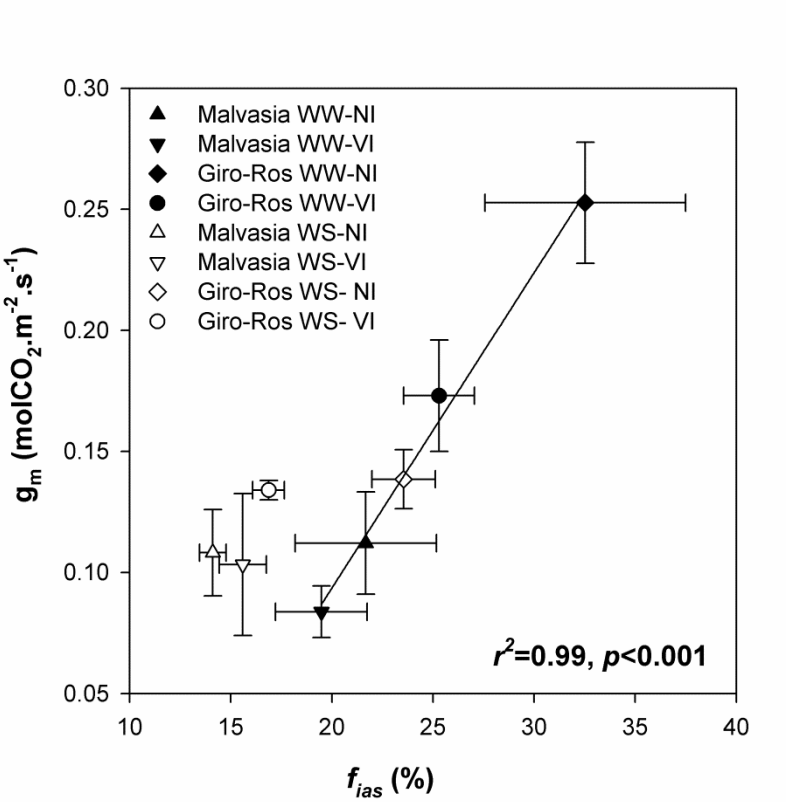

Figure 5. The relationship between mesophyll diffusion conductance (g_m) and leaf porosity in Noninfected (NI) and virus-infected (VI) Vitis vinifera plants (Malvasia de Banyalbufar and Giró Ros) under water stress (WS) and well-watered (WW) conditions. Values are means ± SE of three replicates for anatomical components and 5 replicates for g_m. The data were fitted by linear regression.

Table 4. Photosynthetic pigments; total chlorophyll (Chltotal), total carotenoid content; total soluble proteins (TSP) and Rubisco concentration/ TSP in non-infected and virus infected plants under well-watered and water stress conditions in both cultivars. Values are means of 5 replicates ± S.E. Superscript letters denote significant differences ($P < 0.05$) among different treatments in each variety based on Duncan's test for each of the parameters.

Parameters	**Malvasia de Banyalbufar**				**Giró Ros**			
	Control		**Water-stress**		**Control**		**Water-stress**	
	NI	**VI**	**NI**	**VI**	**NI**	**VI**	**NI**	**VI**
TSP(mg/g)	$51.4^{a} \pm 3.4$	$40.9^{a} \pm 2.8$	$51.3^{a} \pm 5.0$	$42.3^{a} \pm 11.7$	$37.2^{a} \pm 4.3$	$32.0^{a} \pm 5.3$	$36.3^{a} \pm 5.3$	$41.3^{a} \pm 2.5$
[Rub]/[TSP] (mg mg-1)	$0.13^{a} \pm 0.01$	$0.16^{ab} \pm 0.01$	$0.23^{b} \pm 0.01$	$0.19^{ab} \pm 0.02$	$0.18^{ab} \pm 0.03$	$0.17^{ab} \pm 0.02$	$0.19^{ab} \pm 0.02$	$0.15^{a} \pm 0.03$
Chltotal (mg g-1)	$2.1^{a} \pm 0.5$	$1.4^{a} \pm 0.2$	$2.1^{a} \pm 0.2$	$2.5^{a} \pm 0.3$	$1.6^{a} \pm 0.2$	$1.4^{a} \pm 0.2$	$2.4^{a} \pm 0.5$	$2.8^{a} \pm 0.5$
Cartot(mg g-1)	$0.40^{a} \pm 0.10$	$0.30^{a} \pm 0.05$	$0.33^{a} \pm 0.08$	$0.46^{a} \pm 0.08$	$0.38^{a} \pm 0.04$	$0.35^{a} \pm 0.05$	$0.54^{a} \pm 0.09$	$0.63^{a} \pm 0.09$
Chla/b	$2.8^{a} \pm 0.2$	$3.4^{a} \pm 0.8$	$3.7^{a} \pm 0.3$	$3.4^{a} \pm 0.3$	$3.5^{a} \pm 0.3$	$3.5^{a} \pm 0.4$	$3.9^{a} \pm 0.1$	$3.8^{a} \pm 0.1$

Table 5. Mean ± SE values of leaf thickness (*T*L), leaf mass per area (M_A), leaf density (D_L) thickness of mesophyll layers (Spongy and Palisade) and mesophyll porosity (f_{ias}) in both varieties.

	Treatments	Malvasia de Banyalbufar				Giró Ros			
		Control		Water stress		Control		Water stress	
		NI	VI	NI	VI	NI	VI	NI	VI
	T_L (µm)	$150.3^{b} \pm 1.6$	$185.6^{d} \pm 0.9$	$155.2^{b} \pm 1.4$	$134.3^{a} \pm 1.6$	$189.5^{d} \pm 2.2$	$198.5^{e} \pm 1.9$	$201.6^{e} \pm 2.3$	$178.1^{c} \pm 9.6$
	M_A (g/m^2)	$53.4^{d} \pm 3.8$	$69.8^{e} \pm 5.7$	$53.6^{cd} \pm 0.6$	$49.0^{d} \pm 0.8$	$45.1^{c} \pm 0.5$	$43.2^{bc} \pm 1.5$	$34.1^{ab} \pm 2.5$	$27.4^{ab} \pm 0.7$
	D_L (g/cm^3)	$0.35^{d} \pm 0.00$	$0.38^{e} \pm 0.00$	$0.35^{d} \pm 0.00$	$0.40^{e} \pm 0.00$	$0.24^{b} \pm 0.00$	$0.22^{b} \pm 0.0$	$0.17^{a} \pm 0.00$	$0.27^{c} \pm 0.03$
Mesophyll Thickness	Spongy	$64.1^{ab} \pm 0.9$	$89.8^{d} \pm 1.6$	$73.2^{c} \pm 1.3$	$57.6^{a} \pm 1.4$	$97.9^{e} \pm 1.8$	$102.0^{e} \pm 1.7$	$107.4^{e} \pm 4.1$	$70.8^{bc} \pm 4.8$
	Palisade	$62.9^{c} \pm 1.24$	$69.6^{d} \pm 1.75$	$56.5^{b} \pm 0.87$	$47.8^{a} \pm 0.78$	$68.1^{cd} \pm 1.1$	$69.7^{d} \pm 0.8$	$80.0^{e} \pm 2.5$	$52.4^{ab} \pm 3.4$
	f_{ias} (%)	$21.6^{bc} \pm 3.4$	$19.4^{abc} \pm 2.2$	$14.1^{a} \pm 0.6$	$15.6^{a} \pm 1.1$	$32.5^{d} \pm 4.9$	$25.3^{c} \pm 1.7$	$23.9^{c} \pm 1.5$	$16.4^{ab} \pm 0.7$

[1]values are means of 3 replicates. [2]Superscript letters denote significant differences ($P < 0.05$) among different treatments in each variety based on Duncan's test for each of the traits

DISCUSSION

During two consecutive years, the single and combined stresses of water deficit and virus infection were analysed in grapevine. The results showed that photosynthesis (A_N) in both cultivars decreased by virus infection. Previous study by McDowel (2011) showed that carbon metabolism and plants hydraulic are interconnected, thereby virus effects on A_N could be explained by its effects on hydraulic and the different leaf conductance's, g_s and g_m.

Concerning stomatal conductance, our results suggest that g_s was the main factor for decreasing photosynthesis in VI-Malvasia de Banyalbufar under well-watered conditions. The decrease in g_s in VI plants and under WW condition is apparently not a consequence of the plant water status, since leaf water potential at predawn did not differ between NI and VI plants (-0.15 and -0.17 MPa, respectively). The same results were observed by Endeshaw et al. (2014), who indicated that stomatal closure in VI was independent of vine water stress, but might indicate the multiplication of VI in the phloem vessels. The observed differences between Malvasia and Giró Ros could be explained by the significantly higher virus amount in Malvasia irrigated plants than in Giró Ros (Fig 2). These results seem to corroborate the recently reported by our group in Malvasia (Montero et al., 2016c), showing that g_s was invariable at low virus concentration.

On the other hand, low levels of A_N due to the virus infection in Giró Ros which were closely related to decreases in g_m. Similar results were obtained in highly infected Malvasia cv. plants where CO_2 diffusion through the mesophyll was an important factor limiting photosynthesis (Sampol et al., 2003). CO_2 diffusion reductions were also reported in other grapevines varieties infected by grapevine leafroll virus (Cabaleiro et al., 1999; Bertamini et al., 2004, 2005; Moutinho-Pereira et al., 2012; Endeshaw et al., 2014, Montero et al. 2016a).

The observed differences in g_m between both cultivars might result from variation in their leaf anatomical traits which can affect effective diffusion path length and area for diffusion of CO_2, as g_m and (f_{ias}) were higher in Giró Ros than Malvasia de Banyalbufar. Interestingly, a positive relationship between g_m and leaf porosity was observed in the present study under well-watered condition. As a consequence, the thicker leaves in infected plants have a lower proportion of mesophyll cell surface area

exposed to intercellular air spaces per unit leaf surface area (Moutinho-Pereira et al., 2012), making CO_2 diffusion more difficult and limiting photosynthesis. This result was in accordance with previous study on g_m reductions with seven grapevine cultivars in well-irrigated plants (Tomas et al., 2014).

Our results show that different morphological modifications took place also in response to virus infection (Table 5). According to Hoefert et al. (1967), in leafroll-infected leaves starch accumulation occurs; specifically in spongy parenchyma, palisade tissue and in the starch sheath of midrib bundles. Moreover, it's has been reported that viral, bacterial and fungal infection induced the callose accumulation in the plasmodesmata and then, the metabolic translocation impairment in the leaves (Kathiria et al., 2010).

The effect of VI in D_L could be due to flavonoids, lignin and phenolics compounds accumulation in grapevine leaves, as reported by Gambino et al. (2012) and Boubakri et al. (2013) in grapevine infected by rupestris stem pitting-associated virus (RSPaV) and *Plasmopara viticola* (fungal), respectively. Moreover, our results have shown that VI effect in anatomical parameters (T_L, M_A, $D_{L)}$ under WW were more pronounced in Malvasia than Giró Ros (Table. 5). Indeed, the high virus concentration in Malvasia as compared with Giró Ros could be the main factor explaining those anatomical differences observed between cultivars (Table 5, Fig 2).

Despite the CO_2 diffusional effects, our results suggested that decreased carboxylation capacity and the maximum electron transport rate were also limiting photosynthesis in virus-infected plants. Reductions in V_{cmax} and J_{max} were previously reported in response to virus infection in grapevines (Moutinho-Pereira et al., 2012; Endeshaw et al., 2014). The non-alteration of Rubisco content suggested that the decrease in V_{cmax} is due to reduced Rubisco activity. Decreases in Rubisco activity in VI infected plants were previously reported in Malvasia by Sampol et al. (2003).

In respect of the water stress effects, moderate water deficit resulted in a closure of stomata and a decrease of diffusion of CO_2 inside the leaf accompanied by a decrease in A_N. These results are in accordance with previous studies where g_m decreases in response to water stress (Grassi and Magnani, 2005; Ripley et al., 2007; Flexas et al.,

2009; Galmés et al., 2011; Tomas et al., 2014). The two leaf conductance parameters (g_s and g_m) were correlated with A_N reduction in Malvasia de Banyalbufar and Giró Ros respectively, with g_s being the major determinant of photosynthetic limitation as also observed in other studies on grapevine.

Beside great change in photosynthetic parameters during water stress, decreased photosynthetic capacity as reflected by both decreased V_{cmax} and J_{max} has been observed in NI of Malvasia during first experimental year. On the other hand, in the second experimental year we didn't find differences in V_{cmax} in both cultivars, as observed by other studies (Galle et al., 2011; De Souza et al., 2005). In parallel, photosynthetic pigments, TSP and Rubisco concentration remained invariable under this condition. These observations confirmed previous results on RuBP content and Rubisco activity which usually remained constant under moderated stress and declined only under severe stress (Bota et al., 2004).

The responses of plants to simultaneous abiotic and biotic stresses are highly complex (Garrett et al., 2006). In some cases, water stress causes more severe symptoms in bean and sorghum plants infected with the fungal pathogen (Mayek-Pérez et al., 2002). Also, higher water stress effect was reported in grape plants infected by the bacterial pathogen Xyllela fastidiosa (McElrone et al., 2001). Contrariwise, the results from other study, showed that plants infected with several RNA viruses exhibited better tolerance and survival to drought (Xu et al., 2008). A recent study reported increases of g_s and A_N in Grapevine rupestris stem pitting-associated virus **(**GRSPaV)-infected plants during progressive water stress (Pantaleo et al., 2016). In the present study, a reduction in all physiological parameters (A_N, g_s and g_m) has been shown under combined virus infection and water stress compared with NI control plants; except in Malvasia cv. in 2013 when g_m seemed unaffected. The limitations analysis revealed that effectively the combined stress tends to increase more total photosynthetic limitation in both cultivars as compared with control treatment (WW-NI) (Fig.3). The photosynthetic reduction by virus and water stress could be explained by impairment metabolic translocation between source-sink, inhibition of Rubisco and other photosynthetic enzymes (Shalitin and Wolf, 2000; Bertamini et al., 2004). The observed decrease of g_m under combined stress seems to be related to the increase in D_L and decrease in intercellular air space according to Evans et al. (1994). Effectively, D_L increased by

12% under combined stress, while this parameter increased just by 2% in individual stress (WW-VI). Moreover, leaf porosity decrease by 40% under combined stress, instead of 16% and 30% under individual stress (VI and water stress respectively). However, no significant differences were observed between NI and VI under water stress. This lack of virus infection effect may be related to low virus concentration in water stressed plants (Fig. 2). Further investigations are recommended to confirm this, by introducing different levels of infection.

CONCLUSION

GLRaV-3 infection changed physiological and anatomical parameters in both cultivars, being Malvasia more affected than Giró Ros under well-watered conditions. The photosynthesis reduction was due to a lower CO_2 diffusion but also to metabolic processes. In both cultivars, moderate water stress resulted in a diffusional limitation to CO_2, with g_s being the main factor affecting photosynthesis. The present results show that the interaction GLRaV-3-water stress induced reductions in several parameters: A_N, g_s. V_{cmax} and J_{max}. However, no virus effects were observed when only water stressed plants were taking into account. Some differences observed between cultivars can be attributed to the genetic variability in plant-pathogen interactions, but virus concentration could be a main key to explain the virus effect under well-watered and water stress conditions.

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Chapter 4

COMBINED EFFECT OF VIRUS INFECTION AND WATER STRESS ON WATER FLOW AND WATER ECONOMY IN GRAPEVINES

COMBINED EFFECT OF VIRUS INFECTION AND WATER STRESS ON WATER FLOW AND WATER ECONOMY IN GRAPEVINES

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ABSTRACT

Water limitation is one of the major threats affecting grapevine production. Thus, improving water use efficiency (WUE) is crucial for a sustainable viticulture industry in Mediterranean regions. Under field conditions, water stress is often combined with viral infections since those are present in major grape-growing areas worldwide. *Grapevine leafroll associated virus-3* (GLRaV-3) is one of the most important viruses, affecting grapevines. Indeed, the optimization of water use in a real context of virus infection is an important topic that needs to be understood. In this work, we have focused our attention on determining the interaction of biotic and abiotic stresses on WUE and hydraulic conductance (K_h) parameters in two white grapevine cultivars (Malvasia de Banyalbufar and Giró Ros).

Under well-watered (WW) conditions, virus infection (VI) provokes a strong reduction ($p<0.001$) in $K_{petiole}$ in both cultivars, however K_{leaf} was only reduced in Malvasia de Banyalbufar. Moreover, the presence of virus also reduced whole-plant hydraulic conductance (K_{plant}) in 2013 and 2014 for Malvasia de Banyalbufar and in 2014 for Giró Ros. Thus, the effect of VI on water flow might explain the imposed stomatal limitation. Under water stress conditions (WS), the virus effect on K_{plant} was negligible, because of the bigger effect of WS than VI.

Whole plant water use efficiency (WUE_{wp}) was not affected by the presence of virus neither under WW nor under WS conditions, indicating that plants may adjust their physiology to counteract the virus infection by maintaining a tight stomatal control and by sustaining a balanced carbon change.

Abbreviations: GFkV, grapevine fleck virus; GFLV, Grapevine fanleaf virus; GLRaVs, Grapevine leafroll-associated viruses; HCFM, Hydraulic Conductance Flow Meter; K_h, hydraulic conductance; RT-PCR, reverse transcription-polymerase chain reaction; WUE, water use efficiency.

INTRODUCTION

Reducing water use for irrigation and increasing water use efficiency (WUE) has become a major priority in agricultural and viticulture research (Morison et al., 2008). Recently, considerable efforts have been made to study the genetic control over WUE in plants and much attention has been devoted to understanding the physiological mechanisms that control this character. There is a large list of regulatory mechanisms implicated in the control of WUE. Among them, plant and leaf hydraulic conductance (K_{plant} and K_{leaf}, respectively) and stomatal conductance (g_s), as an important physiological parameter controlling hydraulic conductance (K_h) (Lovisolo et al. 2008, Pou et al. 2012; Martorell et al. 2015a, 2015b), have gained most of the attention.

In light of the pressure imposed by climate change, several strategies have been proposed to improve WUE in irrigated and rain-fed agriculture by plant breeding (Turner 2004, Cattivelli et al. 2008). However, the effects of plant pathogens on total WUE have not been taken into account. Since evidences suggest that climate change will also expand the host range of pathogens with increased chances of virulent strain development (Garrett et al. 2006), we should consider the possibility that those pathogens may be affecting the physiology of host plants in many different ways, as have been previously described in several works (Goodman et al. 1986, Berger et al. 2007, Barón et al. 2012).

At the leaf level, a range of foliar pathogens have been shown to disrupt the normal stomatal regulation, photosynthesis and transpiration rates (Gondo M. 1953, Lindsey and Gudanskas 1974, Erion et al. 2012). Thus, deleterious effects on instantaneous WUE (WUE_{int}) have been demonstrated in a number of pathosystems (Grimmer et al. 2012), like the fungi *Uncinula nectar* (Powdery Mildew) affecting grapevine leaves (Lakso et al. 1982). Additionally, the effects of plant pathogens on the xylem water transport have been widely illustrated in grapevine and cucumber (McErlone et al. 2003, Choat et al. 2009, Wang et al. 2014), generally showing reductions in petiole hydraulic conductance, stomatal conductance, photosynthesis and leaf water potential.

Nowadays, understanding the effect of pathogens on water relations when plants are under water stress is becoming an increasing challenge for researchers (Atkinson and Urwin 2012). There are studies that discuss the idea that water stress enhances symptom severity along the stem in plants infected with xylem limited bacteria *Xylella fastidiosa* (Xf) (McElrone et al. 2001, 2003). However, other works pointed out the idea that the interaction of plants with pathogens can also be beneficial to improve abiotic stress tolerance (Reusche et al. 2012, Pantaleo et al. 2016).

In grapevines (*Vitis vinifera* L.), *Grapevine leafroll associated virus-3* (GLRaV-3) is one of the most worldwide spread viruses being described as a very damaging disease in comparison with other diseases (Naidu et al. 2008). In this sense, the effect of this virus in water use is a core subject of interest for a sustainable viticulture. Recently, it has been shown that grapevine leaf-roll disease caused by GLRaV-1 and GLRaV-3 virus, increases the intrinsic water use efficiency (A/g_s) in Touriga Nacional cv. under field condition (Moutinho-Pereira et al. 2012). Nevertheless, studies revealing the effects of specific phloem limited virus on water transport, as is the case for GLRaV-3, are scarce and still far from being understood (Moreshet et al. 1998, Pantaleo et al. 2016).

The combined effect of water stress and GLRaV-3 in grapevine on leaf anatomy and gas exchange has been recently studied in our group (El Aou-ouad et al. 2016), however the effect on hydraulic conductance and water use efficiency have yet to be studied. Therefore, the aim of this work is to investigate the combined effect of biotic (GLRaV-3) and abiotic (drought) stresses on leaf and whole plant hydraulic conductance; and secondly to assess if the observed effect of combined stress on hydraulic and gas exchange parameters are reflected in WUE in two different grapevine cultivars, Malvasia de Banyalbufar and Giró Ros under well-watered and water stress condition.

MATERIAL AND METHODS

Plant Material and treatments

The experiments were carried out at the experimental field of the Universitat de les Illes Balears (Palma de Mallorca. Balearic Island, Spain) in two successive summers

(2013 and 2014; from July 8th to August 13th). Both experiments were performed using two white grapevine cultivars: Malvasia of Banyalbufar and Giró Ros.

Plants were obtained by direct rooting of 0.2 m cuttings of dormant canes selected from mother plants growing under field conditions in a twelve-year-old experimental vineyard sited at IRFAP center (Institut de Recerca i Formació Agrària i Pesquera. Conselleria d'Agricultura Medi Ambient i Territori, Palma de Mallorca, Balearic Island, Spain). Cuttings were collected from asymptomatic infected vines (VI), and non-infected vines (NI). Rooting was induced by using indolbutyric acid (IBA. 2g L^{-1}) and plants were maintained in a greenhouse under controlled conditions: Soil temperature 26-28 ºC, air temperature 23 ± 0.1 ºC and air humidity about 80%. When cuttings presented 4-5 expanded leaves, they were transplanted into pots and grown outdoors in 10 L pots filled with organic substrate and perlite mixture (5:1). They were irrigated daily from May until the start of the experiment and supplemented three times per week, with 50% organic-mineral fertilizer NPK containing (%): N. 5; P_2O_5. 8; K_2O. 15; MgO. 2; organic C. 17.4. humic acid. 5; SO_3. 15; Fe. 1; Zn $2x10^{-3}$; Mn $1x10^{-2}$.

A layer of perlite was added to the surface of each pot to reduce soil evaporation. Water stress treatment was defined by the leaf maximum daily stomatal conductance (g_s) according to Medrano et al. (2002). In both experiments, five plants per treatments of Non-infected (NI) and GLRaV-3 (VI) infected plants were subjected to two irrigation regimes: field capacity (g_s> 200 mmol H_2O m^{-2} s^{-1}) and moderate drought (50 <g_s <100 mmol H_2O m^{-2} s^{-1}). Once the desired g_s values were achieved (typically 4-5 days after water withholding), pots were weighed daily in the evening and the amount of water consumed was replaced to maintain the same level of drought for four weeks. Control plants were maintained at field capacity throughout the experiments.

Plant water status and climatic conditions

Predawn (Ψ_{PD}) and midday leaf water potential (Ψ_{MD}) were measured in five fully expanded leaves per cultivar and treatment, at the end of July, with a Scholander pressure chamber (Soil moisture Equipment Corp. Santa Barbara. CA).

Climatic data (mean T (daily mean air temperature), max T (daily maximum temperature), min T (daily minimum temperature), accumulated evapotranspiration

(ETP accum), relative humidity and rainfall) were obtained from a weather station 7450 Groweather (DAVIS instruments Corp, Hayward. California. USA), located in the experimental field at the *Universitat de les Illes Balears* (Mallorca. Spain).

Pressure-volume (P-V) Curves

Pressure-volume curves were carried out on randomly selected mature and sun exposed leaves (six to seven leaf selected based in their position in the shoot tip) according to Tyree and Richter (1981) and Alsina et al. (2007) during 2013 experiment. Leaves were collected the day before measuring and were left to rehydrate overnight before P-V determination. Leaf water potential (Ψ_{leaf}) was measured using a pressure chamber (Soil moisture Equipment. Corp., Santa Barbara, CA, USA) and leaf weight was measured with an analytical balance (Kern ABT320-4M, precision of 0.0001 g) during the slow dehydration process in the laboratory. Turgor loss point is the inflection point of the $1/\Psi_{leaf}$ versus relative water content (RWC) curve. The bulk leaf modulus of elasticity (ε) was estimated as the slope of turgor potential (Ψ_p) versus RWC through the phase from full turgor to turgor loss point ($\Psi_{\pi.FT}$) in five replicates per treatment and cultivar. All measurements were performed at a constant temperature. The fitting method proposed by Sack & Pasquet-Kok (2011) was used to fit the P-V curves.

Virus detection and quantification

The presence or absence of GLRaV-3 was verified in mother plants by enzyme-linked immunosorbent assay (ELISA) (Clark and Adams, 1977) using commercial coating and conjugate antibody preparations (Bioreba AG, Reinach, Switzerland). Even more, the infection level was quantified by real-time PCR reactions in five different plants per treatment as previously described in El Aou-ouad et al. (2016).

Leaf gas exchange measurements

Instantaneous gas exchange measurements were made at the end of July on five young and fully expanded leaves (sixth leaf selected based in their position in the stem tip) per treatment in five different plants at mid-day using an open gas exchange system (Li-6400; Li-Cor. Inc., Lincoln. NE) equipped with a leaf chamber fluorometer (Li-

6400-40; LI-COR Inc.). Measurements of net CO_2 assimilation (A_N), stomatal conductance (g_s), transpiration (E) and internal CO_2 concentration (C_i) were performed at saturating light (1500 µmol m^{-2} s^{-1}) achieved with the red LED lamp of the system with an additional 10% blue light to maximize stomatal opening. CO_2 concentration in the leaf chamber (C_a) was set at 400 µmol CO_2 mol^{-1}air in the cuvette and the relative humidity of the incoming air ranged between 40 and 60%. Block temperature was maintained at 30ºC.

Intrinsic WUE (A_N/g_s) was calculated as the ratio between A_N and g_s while instantaneous WUE (A_N/E) was obtained from the ratio between A_N and E.

Carbon isotope composition in leaf dry matter

Long-term water use efficiency was assessed by measuring carbon isotope composition ($\delta^{13}C$). Five young leaves per cultivar and treatment from different plants were taken at the end of the experiment and oven-dried for 48h at 60ºC. Dried leaves were ground into powder and subsamples of 2 mg were analysed for isotope ratio ($\delta^{13}C$). Samples were combusted in an elemental analyser (Carlo-Erba, Rodano, Italy). CO_2 was separated by chromatography and directly injected into a continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta Plus. Bremen. Germany). Peach leaf standards (NIST 1547) were run every eight samples. $\delta^{13}C$ was calculated as: $\delta^{13}C$ sample (‰) = ((R sample/R standard) –1)·1000 (Farquhar and Richards 1984). $\delta^{13}C$ values are referred to a Pee Dee Belemnite standard.

WUE at whole-plant level

Whole plant WUE was estimated in two consecutive years (2013-2014) as follows: At the beginning of the experiment four plants per variety were harvested to determine initial whole plant biomass. Similarly, five plants per cultivar and treatment were harvested at the end of the experiment. Leaves, stems and roots from each plant were separated and dried in an oven at 60ºC to obtain their dry weight. Total biomass increment was obtained from the difference between whole plant dry weight at the end and the beginning of the experiment.

Total plant water consumed over the 4 weeks period was estimated from the sum of the daily water consumption previously described.

Whole plant water use efficiency was determined as follows:

$$WUE_{WP}\ (g\ L^{-1}) = \frac{(\text{dry weight final biomass - dry weight initial biomass})}{\text{total water consumed}}$$

Whole-plant hydraulic conductance

Whole plant hydraulic conductance (Kh_{plant}) was calculated by the Ohm's law analogy for the soil-plant-atmosphere continuum (Lovisolo et al. 2002) in five plants per cultivar and treatment:

$$E = Kh_{plant} \times (\Psi_{MD} - \Psi soil).$$

Where E, Kh_{plant}, Ψ_{leaf} and Ψ_{soil} represent transpiration rate, whole-plant hydraulic conductivity, leaf water potential and soil water potential respectively. Ψ_{PD} was taken as a proxy for Ψ_{soil} and Ψ_{MD} was taken as Ψ_{leaf}.

Leaf and Lamina hydraulic conductance

Maximum leaf hydraulic conductance on a surface area basis (K_{leaf}, mmol m^{-2} s^{-1} MPa^{1}) was determined in four plants per cultivar and treatment, only in the second experimental year, using a high-pressure flow meter (HPFM-XP; Dynamax Inc. Houston TX. USA) described in detail by Tyree et al. (1995). Unfortunately, it was not possible to measure the Malvasia de Banylbufar cultivar under water stress, because of technical issues during the experiment. Detached leaves were excised under water and allowed to reach a transpirational steady-state while attached to a flow meter via the petiole using compression fittings. For each measurement 15 mM KCl solution filtered at 0.1 μm, was forced into the leaves at a constant pressure (P; MP) up to 0.4 MPa, while measuring instantaneous flow (F; Kg s^{-1}) every 8s.

Corresponding hydraulic conductance (*K*) were computed as *K*= F/P. Quasi Steady-State mode was used for each measurement. K decreased during the early phases of measurements as the likely effect of progressive infiltration of leaf air spaces, and reached stable values after 25–30 min. After *K* was recorded, leaf blades were removed using a fresh razor blade. The hydraulic conductance of the petiole ($K_{petiole}$) was

similarly measured and the lamina hydraulic conductance (K_{lamina}) (K_{lamina}=1/R) was calculated as: $1/K_{lamina} = (1/K_{leaf}) – (1/K_{petiole})$

During measurements, leaf temperature was monitored by a thermocouple and maintained between 20 °C and 25 °C by adding water uniformly across the leaf blade. The leaf conductance values were standardized for the effects on temperature on the viscosity of water by correcting K_{leaf} to a value for 25 °C (Yang and Tyree 1994) according to manufacturer's instructions:

$$K_{corrected} = K\,(0.554 + 0.0225\,T) / (0.554 + 0.0225\,T^*)$$

Where K is the uncorrected conductance, T is the temperature at which K was measured and T* is the temperature at which the HPFM was calibrated.

After each experiment, projected leaf areas (LA; m^2) were measured using Image J (ImageJ; Wayne Rasband/NIH, Bethesda, Maryland, USA) and leaf and lamina maximum hydraulic conductance on a surface area basis were calculated (K_{leaf} and K_{lamina}, respectively; mmol s^{-1} MPa^{-1} m^{-2}). Leaf and lamina hydraulic conductance were measured in four replicates per cultivar and treatment.

Abscisic acid, jasmonic acid and salicylic acid quantification

In the first year (2013), young fully expanded and sun exposed leaves were excised from five different plants and treatment and xylem exudation was collected after applying sufficient pressure with a leaf pressure chamber (Soil Moisture Equipment Corp. Santa Barbara. CA. USA). After discarding the first exudation, sap was collected and immediately submerged in liquid nitrogen and kept at –80 ºC. The analysis of abscissic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) phytohormones were performed by HPLC-ESI-MS/MS using an Agilent 1200 Series HPLC system (Agilent. Santa Clara. California. USA) coupled to a 3200 QTRAP (Applied Biosystems. California. USA) at the IVICAM (Instituto de la Vid y el Vino de Castilla-La Mancha)

Statistical analysis

Regression coefficients, correlations and box plots were obtained using Sigma Plot 10.0 software package (Systat; Chicago, IL, USA). Analysis of variance (Two-way ANOVA) was performed to reveal differences among treatments in each cultivar and experimental year in the studied parameters. Differences between means were revealed by Duncan analyses ($P < 0.05$), performed with R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

RESULTS

Climatic conditions and plant water status

In general, the climatic variables were similar for both years (Supplementary table S1). No significant differences in mean air and maximum temperatures were reordered between years. In addition, VPD and accumulated evapotranspiration (ETP_{accum}) were also similar for both experimental years (Supplementary Table S1).

As expected, in both cultivars, water withholding significantly reduced Ψ_{Pd} in 2013 and 2014. However, virus infection (VI) didn't reflect any change in Ψ_{Pd} either under well-watered (WW) and water stress (WS) conditions in both years (2013-2014) (Table 1). During 2013, osmotic potential at full turgor ($\Psi_{\pi.FT}$) and modulus of the elasticity (ε) were obtained from pressure-volume curves. No differences in $\Psi_{\pi.FT}$ were observed between treatments and cultivars. In general, values were similar among treatments in each cultivar. In Malvasia de Banyalbufar, WS imposition in NI plants increased ε, whereas the presence of virus did not affect this parameter neither in WW or WS. In Giró Ros, ε was not statistically different among treatments.

Table. 1 Predawn leaf water potential (Ψ_{Pd}), osmotic potential at full turgor ($\Psi_{\pi.FT}$) and modulus of elasticity (ε). Values are mean of four replicates ± standard errors. Letters denote significant differences between treatments in each cultivar and experimental year ($P < 0.05$) according to Duncan's test.

		2013			2014
	Treatments	**Ψ_{Pd} (MPa)**	**$\Psi_{\pi.FT}$ (MPa)**	**ε (MPa)**	**Ψ_{Pd} (MPa)**
Malvasia	WW-NI	- 0.15 ± 0.02^{a}	-1.03 ± 0.45^{ab}	10.33 ± 0.90^{b}	- 0.16 ± 0.03^{a}
	WW-VI	- 0.17 ± 0.02^{a}	-1.09 ± 0.06^{b}	9.85 ± 0.97^{b}	- 0.21 ± 0.02^{a}
	WS-NI	- 0.19 ± 0.01^{b}	-1.16 ± 0.15^{b}	15.32 ± 1.38^{a}	- 0.43 ± 0.03^{c}
	WS-VI	- 0.23 ± 0.01^{b}	-0.75 ± 0.02^{a}	8.38 ± 1.08^{b}	- 0.34 ± 0.02^{b}
	$T_{abiotic}$	***	ns	ns	***
	T_{biotic}	ns	.	*	ns
	T_{biotic} vs $T_{abiotic}$	ns	*	*	*
Giró-Ros	WW-NI	-0.13 ± 0.01^{a}	-1.03 ± 0.10^{a}	14.42 ± 1.14^{a}	-0.29 ± 0.02^{a}
	WW-VI	-0.14 ± 0.00^{a}	-1.37 ± 0.16^{a}	17.11 ± 1.83^{a}	-0.31 ± 0.00^{a}
	WS-NI	- 0.23 ± 0.01^{b}	-0.92 ± 0.05^{a}	14.60 ± 0.51^{a}	-0.55 ± 0.01^{b}
	WS-VI	- 0.22 ± 0.01^{b}	-1.0 ± 0.10^{a}	16.57 ± 2.05^{a}	-0.57 ± 0.03^{b}
	$T_{abiotic}$	***	ns	ns	***
	T_{biotic}	ns	ns	ns	ns
	T_{biotic} vs $T_{abiotic}$	ns	ns	ns	ns

Letters treatment indicates: WW-NI, Well-watered non-infected; WW-VI, Well-watered virus-infected; WS-NI, Water stress non-infected; WS-VI, Water stress virus-infected. (.) Difference marginally significant $P > 0.05$, $P<0.05$ (*), $P<0.01$(**), $P<0.001$(***).

Hydraulic conductance and gas exchange parameters

As a result of water depletion, NI plants showed significant reductions in A_N, g_s, E, and K_{plant} for both cultivars and years (Table 2). The presence of VI under WW conditions, significantly decreased A_N in both cultivars during 2013, but no significant differences were observed in 2014 for this parameter. The reduction of A_N in VI plants was more pronounced in Malvasia de Banyalbufar than in Giró Ros, being around 32 % and 18 % respectively. The A_N reduction in Giró Ros 2013 was not a consequence of g_s reductions. Howerver, the presence of virus resulted in g_s reductions for Malvasia de Banyalbufar 2013 and both cultivars in 2014. Similar results were observed in K_{plant}, being significantly reduced by the presence of VI in both cv. and years (except for Giró Ros 2013).

The combination of stresses resulted in significant reductions of all parameters when compared with WW-NI plants in both cultivars and years (Table 2). The two-way ANOVA revealed no significant interactions between treatments (water stress and virus infection) for most of the parameters. In 2013 the interaction between treatments was significant in Malvasia de Banyalbufarfor E, and in 2014 for g_s and K_{plant} in Malvasia de Banyalbufar.

The obtained relationship g_s vs K_{plant} (Fig. 2) showed that both cultivars followed the same trend, and a unique linear regression was plotted for both of them, obtaining highly significant regression coefficients.

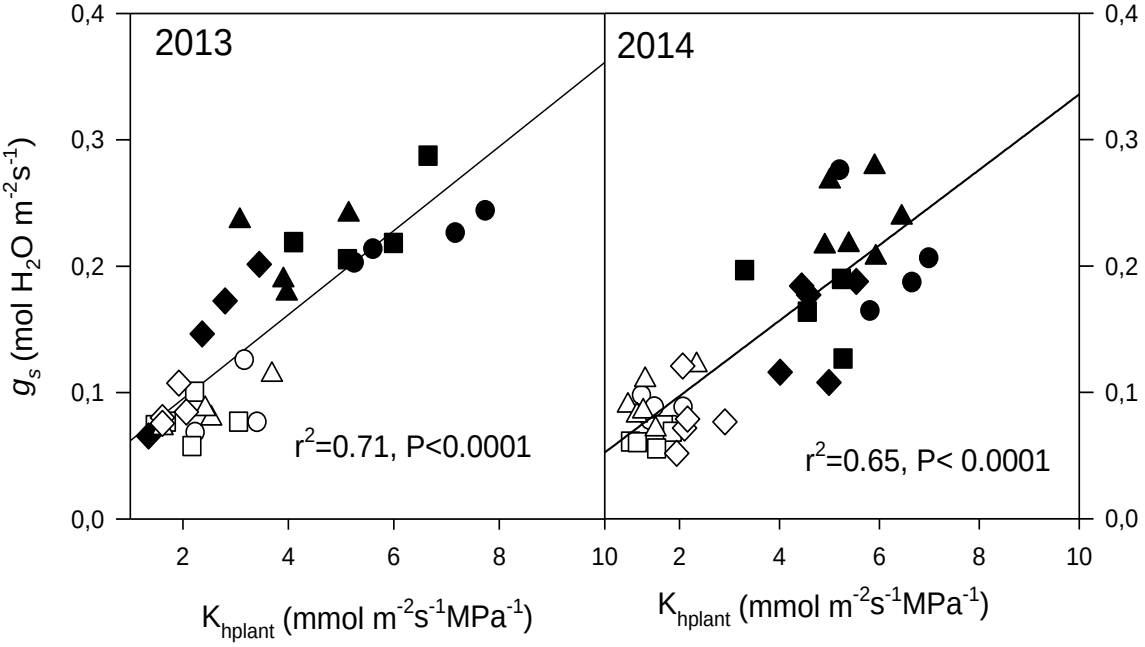

Figure 1 Relationship between plant hydraulic conductance (K_{plant}) and stomatal conductance (g_s) between cultivars Malvasia and Giro-Ros. White colour means values during water stress and Black colour means values during well-watered conditions. Circle, square symbols represent non-infected (NI) and virus-infected (VI), respectively in Giró Ros. Triangle and diamond symbols represent non-infected (NI) and virus-infected (VI), respectively in Malvasia de Banyalbufar. Values are means of 5 replicates ± S.E per cultivar and treatment.

Hormone responses

In both cultivars, [ABA], [SA] and [JA], hormones involved in plant defence response, were determined in NI and VI under WW and WS conditions. In Giró Ros, no significant differences were observed in the hormone content between virus infected and healthy plants under WW or WS conditions. In Malvasia de Banyalbufar, there was

a significant increase of leaf ABA when water depletion was imposed, whereas, JA significantly decreased under WS condition.VI did not affect any of the measured hormones under WW. However, the combined GLRaV-3 infection and water stress (WS-VI) resulted in higher [ABA] and lower [JA] as compared to WW-NI condition, while, SA remained invariable in all treatments (Fig. 1).

Leaf and petiole hydraulic conductivity

Regardless of treatments, the results revealed that leaf hydraulic conductivity (K_{leaf}) was higher in Giró Ros, with almost double K_{leaf} values as compared with Malvasia de Banyalbufar, being 12.1 $mmolm^{-2}$ $s^{-1}MPa^{-1}$ and 7.1 $mmolm^{-2}$ $s^{-1}MPa^{-1}$, respectively. As a result of water depletion, Giró Ros showed a strong reduction in K_{leaf} and $K_{petiole}$, noting in this case, that $K_{petiole}$ experience larger reductions than K_{leaf} (Fig. 2). The presence of GLRaV-3 under WW conditions, strongly decreased K_{leaf} and $K_{petiole}$ in Malvasia. In Giró Ros, although strong reductions were observed in $K_{petiole}$ ($p<0.01$), K_{leaf} was not statistically different between WW-NI and WW-VI. The combined stress (WS-VI), measured in this case just in Giró Ros, resulted in lower K_{leaf} and $K_{petiole}$ as compared with WW-NI. However, no interactive effect was observed if compared with WS-NI (Fig. 3).

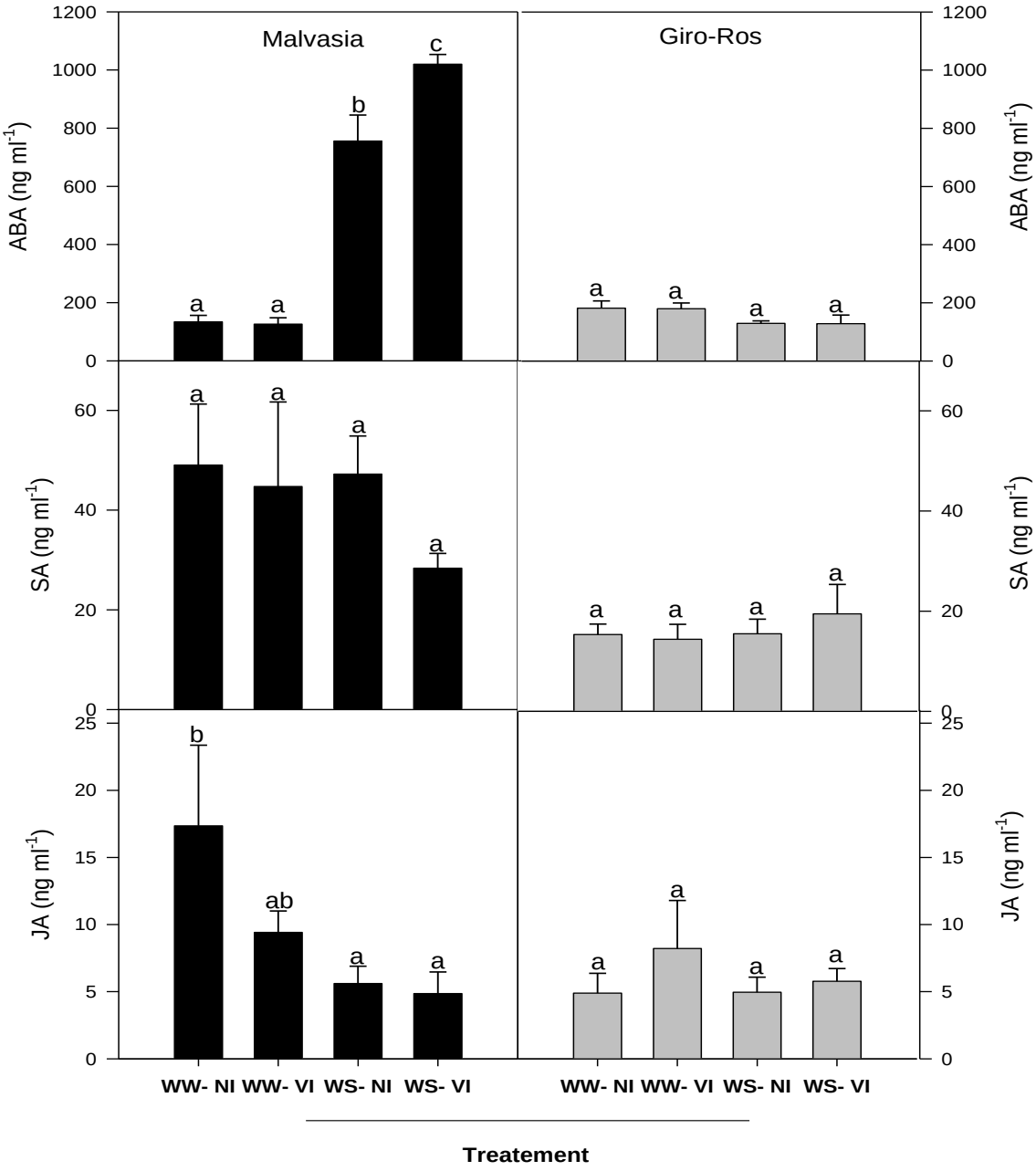

Figure 2 ABA, SA and JA concentration in non-infected (NI) and virus infected (VI) plants of Malvasía de Banyalbufar and Giró Ros cultivars, in July 2013, under water stress (WS) and well-watered (WW) condition. Values are means of 5 replicates ± SE per cultivar and treatment.

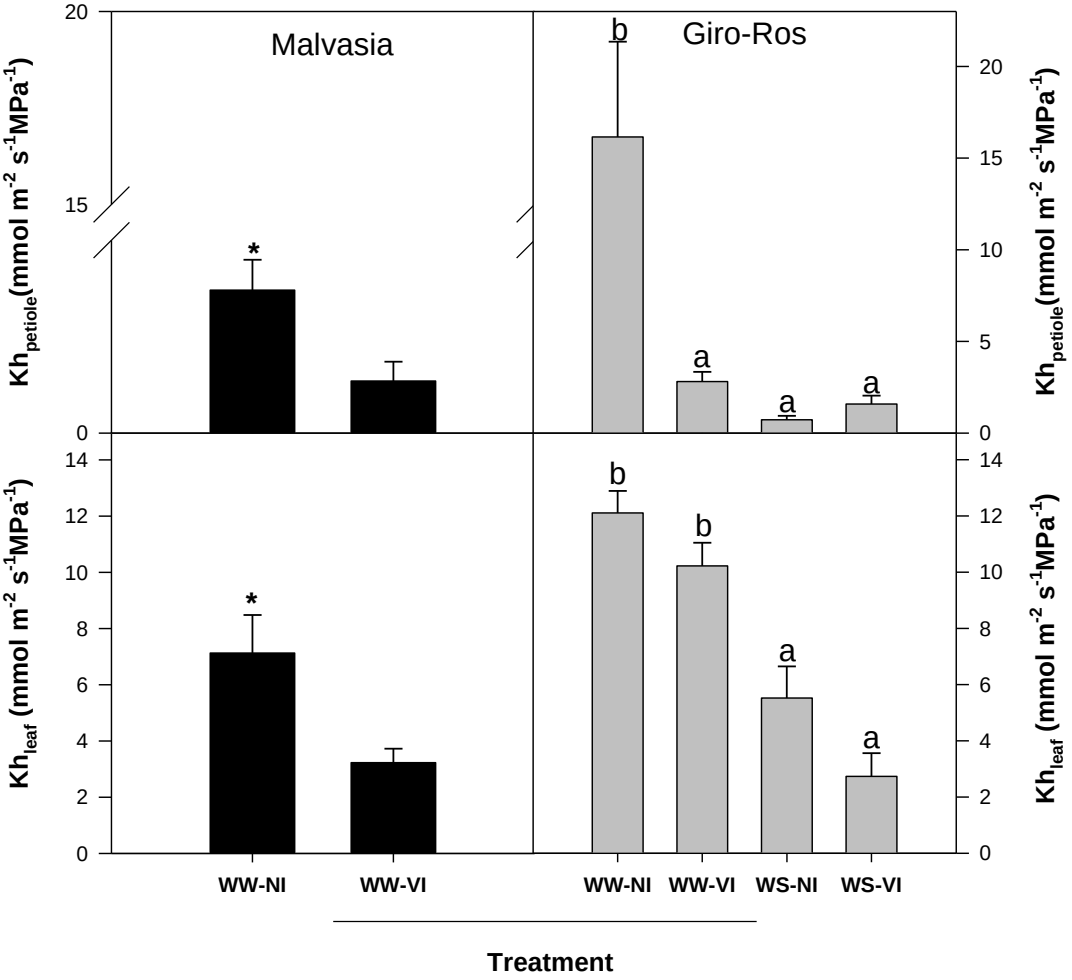

Figure 3 Leaf and lamina hydraulic conductivity in non-infected (NI) and virus-infected (VI) grapevine cvs plants under water stress and well-watered condition in second experimental year (2014). Values are mean of 4 replicates ± S.E per cultivar and treatment.

WUE at leaf and plant levels

Leaf WUE (WUE_{leaf}) was measured instantaneously (A_N/g_s and A_N/E) or at long term ($\delta^{13}C$). Because of water shortage, A_N/g_s and A_N/E have been significantly increased in both cultivars and experimental years, except for A_N/E in 2013 for Malvasia de Banyalbufar, and in 2014 for Giró Ros which remained unaffected, probably due to parallel decrease of A_N and E. In infected plants, under WW conditions, the presence of virus did not affect A_N/g_s nor A_N/E in any of the cultivars and experimental years. The combined stress significantly increased A_N/g_s in both cultivars and experimental years, whereas A_N/E showed the same response as A_N/g_s in Giró Ros

but not in Malvasia de Banyalbufar which remained uninfected. In this case, two-way ANOVA did not revealed any significant interaction between treatments (Table 2).

Leaf $\delta^{13}C$ significantly increased as a consequence of water depletion; except in 2013 for Giró Ros, which showed no significant differences between WW and WS treatments. In WW conditions, leaf $\delta^{13}C$ was not affected by virus infection in any of the cultivars and experimental years. However, this parameter significantly increased in response to the combined stress in both cultivars and experimental years (Table 2). In this case, two-way ANOVA highlight significant interaction between treatment in Giró Ros 2013 and Malvasia de Banyalbufar 2014.

At the whole plant level, neither moderate water stress nor virus infection showed significant changes in WUEWP. Similarly, the combined stress did not significantly increase this parameter, except in 2013 for Giró Ros, being in this case the interaction between treatments significant when doing two-way ANOVA (Table 2).

Table 2: Net photosynthetic rate (A_N), stomatal conductance (g_s), leaf transpiration (E), plant hydraulic conductance (K_{plant}), water use efficiency (WUE) measured at the leaf level (intrinsic water-use efficiency (A_N/g_s), instantaneous water-use efficiency (A_N/E), leaf carbon isotope ratio ($\delta^{13}C$) and plant level (whole-plant WUE (WUE_{WP})), in the two experimental years (2013-2014).Values are mean of five replicates ± standard errors. Letters denote statistic significant differences by Duncan's test among treatments in each cultivar and in each experimental year ($P < 0.05$). Letters treatment indicates: WW-NI, Well-watered non-infected; WW-VI, Well-watered virus-infected; WS-NI, Water stress non-infected; WS-VI, Water stress virus-infected. P<0.05 (*), P<0.01(**), P<0.001(***).

	Treatments	A_N ($\mu molCO_2 m^2 s^{-1}$)	g_s (mol H_2O m^{-2} s^{-1})	E (mmol H_2O m^{-2} S^{-1})	Kh_{plant} (mmolMPa m^{-2} S^{-1})	A_N/g_s (µmol CO_2 /mol H_2O)	A_N/E (µmol CO_2 /mol H_2O)	$\delta^{13}C$ (‰)	WUE_{WP} (g/L)
2013 Malvasia	WW-NI	13.82 ± 0.26^a	0.189 ± 0.016^a	4.16 ± 0.37^a	4.02 ± 0.42^a	73.4 ± 2.5^b	3.1 ± 0.1^a	-29.1 ± 0.3^b	3.65 ± 0.76^a
	WW-VI	9.53 ± 1.00^b	0.130 ± 0.020^b	3.10 ± 0.45^b	2.49 ± 0.44^b	73.7 ± 6.0^b	3.0 ± 0.2^a	-28.7 ± 0.3^b	2.82 ± 0.11^a
	WS-NI	10.37 ± 0.04^b	0.096 ± 0.010^{bc}	2.88 ± 0.35^b	2.56 ± 0.42^b	10.2 ± 13.8^a	3.6 ± 0.7^a	-26.6 ± 0.2^a	5.01 ±0.98^a
	WS-VI	9.21 ± 0.65^b	0.091 ± 0.008^c	3.08 ± 0.42^b	1.80 ± 0.11^b	99.3 ± 7.6^a	2.9 ± 0.1^a	-27.7 ± 0.4^a	4.47 ± 0.98^a
Significance P-values	$T_{abiotic}$	*	***	*	*	**	ns	***	ns
	T_{biotic}	**	**ns**	ns	*	ns	ns	ns	ns
	T_{biotic}vs $T_{abiotic}$	*	ns	*	ns	ns	ns	ns	ns
Giro-Ros	WW-NI	15.28 ± 0.38^a	0.221 ± 0.009^a	6.71 ± 0.52^a	6.46 ± 0.60^a	69.6 ± 1.4^b	2.2 ± 0.1^b	-28.4 ± 0.1^b	2.83 ± 0.18^b
	WW-VI	12.60 ± 0.34^b	0.233 ± 0.019^a	6.23 ± 0.63^a	5.58 ± 0.44^a	55.8 ± 3.2^b	2.1 ± 0.1^b	-28.3 ± 0.1^b	2.61± 0.37^b
	WS-NI	9.46 ± 0.88^c	0.099 ± 0.014^b	2.73 ± 0.32^b	3.55 ± 0.65^b	95.4 ± 4.0^a	3.6 ±0.5^a	-27.7 ± 0.2^b	3.01 ± 0.16^b
	WS-VI	7.82 ± 0.12^c	0.081 ± 0.004^b	2.12 ± 0.14^b	2.12 ± 0.27^b	96.6 ± 7.6^a	3.6 ±0.2^a	-26.6 ± 0.4^a	3.97 ± 0.08^a
Significance P-values	$T_{abiotic}$	***	***	***	***	***	***	***	ns
	T_{biotic}	**	ns	ns	ns	ns	ns	*	ns
	T_{biotic}vs $T_{abiotic}$	ns	ns	ns	ns	ns	ns	*	**
2014 Malvasia	WW-NI	14.06 ± 0.70^a	0.229 ± 0.010^a	5.09 ± 0.24^a	5.59 ± 0.24^a	62.3 ± 5.4^c	2.7 ± 0.3^b	-26.9 ± 0.1^c	1.48 ± 0.25^a
	WW-VI	12.21 ± 0.50^a	0.173 ± 0.016^b	4.06 ± 0.33^a	4.70 ± 0.25^b	72.5 ± 6.0^{bc}	3.0 ± 0.3^b	-26.8 ± 0.1^c	1.79 ± 0.48^a
	WS-NI	8.62 ± 1.01^b	0.089 ± 0.008^c	1.89 ± 0.22^b	1.45 ± 0.24^d	99.7 ±16.3^{ab}	4.6 ±0.5^a	-25.3 ± 0.0^a	2.36 ± 0.68^a
	WS-VI	8.54 ± 0.62^b	0.084 ± 0.010^c	2.53 ± 0.20^b	2.14 ± 0.14^c	107.8 ± 12.6^a	3.4 ±0.1^b	-25.7 ± 0.2^b	2.04 ± 0.33^a
Significance P-values	$T_{abiotic}$	***	***	***	***	**	*	***	ns
	T_{biotic}	ns	**	ns	*	ns	*	ns	ns
	T_{biotic}vs $T_{abiotic}$	ns	*	ns	**	ns	ns	*	ns
Giro-Ros	WW-NI	14.67 ± 1.31^a	0.235 ± 0.030^a	4.29 ± 0.61^a	5.70 ± 0.43^a	63.6 ± 3.8^b	3.6 ± 0.6^{bc}	-27.3 ± 0.0^b	3.10 ± 0.49^a
	WW-VI	11.70 ± 0.76^{ab}	0.161 ± 0.010^b	3.92 ± 0.25^a	4.62 ± 0.36^b	73.9 ± 6.7^b	2.9 ± 0.2^c	-27.2 ± 0.1^b	2.92 ± 0.25^a
	WS-NI	9.21 ± 1.02^{bc}	0.084 ± 0.004^c	1.83 ± 0.22^b	1.70 ± 0.14^c	109.7 ± 11.4^a	5.4 ± 0.8^{ab}	-24.7 ± 0.2^a	5.13 ± 0.76^a
	WS-VI	7.56 ± 0.71^c	0.064 ± 0.003^c	1.36 ± 0.19^b	1.39 ± 0.11^c	117.8 ± 8.3^a	5.9 ± 0.8^a	-25.7 ± 0.1^a	4.51 ± 0.72^a
Significance P-values	$T_{abiotic}$	***	***	***	***	***	ns	***	*
	T_{biotic}	*	*	ns	*	ns	ns	ns	ns
	T_{biotic}vs $T_{abiotic}$	ns	ns	ns	ns	ns	ns	ns	ns

DISCUSSION

The effect of water withholding on water use efficiency (WUE) in grapevines is widely described (Chaves et al. 2007, Flexas et al. 2010, Tomas et al. 2012, Martorell et al. 2015, Medrano et al. 2015; Bota et al. 2015), however the influence of virus on the WUE is an important issue to take into account and is nowadays under debate (Moutinho-Pereira et al. 2012). In this article we intend to study the combined effect of both (biotic and abiotic) factors on plant water relations, concretely, leaf water status, plant and leaf hydraulic conductance (K_{plant} and K_{leaf}, respectively), WUE_{leaf} and WUE_{wp}, trying to elucidate if the combined stress may prompt an additive effect on plant physiological parameters, as it has been previously described in Arabidopsis, barley and others crops (Prasad et al., 2011; Vile et al., 2012; Rollins et al., 2013, Prasch and Sonnewald 2013).

Efects of water stress

Consistent with previous studies, the imposition of water stress resulted in significant decrease in predawn water potential (Ψ_{Pd}) (Tomas et al. 2012, Martorell et al. 2015). Recently, it has been shown that reductions in leaf water potential could be partly explained by osmotic adjustment (Hochberg et al. 2015). However, in the current study, any osmotic adjustment was not observed in either cultivar, suggesting that the response mediated by leaf turgor is not the main parameter explaining the stomatal adjustment. The lack of osmotic adjustment was confirmed by a decrease of elasticity of cell walls (i.e. increase in ε) in Malvasia and by unchangeable leaf ε in Giró Ros, suggesting that in the latter case the adjustment in ε was not an important parameter in driving the response to drought stress (Bartlett et al. 2012, Martorell et al. 2015).

Water withholding significantly decreased A_N, g_s and E in both cultivars. These results were consistent with previous studies reporting the drought effect on grapevine, whether in potted or field grown plants (Tomas et al. 2013, Martorell et al. 2014, 2015, Bota et al. 2015). Presently, it has been suggested that stomatal conductance is primarily regulated by passive hydraulic and then by active (ABA-mediated) mechanisms in drought stressed grapevines (Tombesi et al. 2016). Our results revealed that under WS, ABA content increase only in Malvasia de Banyalbufar, but not in Giró Ros, suggesting

that the regulation of stomatal conductance by ABA is cultivar-dependent (Fig. 2). Different behaviors among grapevine varieties in relation to [ABA], K_{plant} and g_s values were also described by Martorell et al. (2015) for Tempranillo and Grenache.

In the current work WS also resulted in low K_{plant}, confirming the tight coordination between liquid flow conductivity and g_s, whether in grapevine or other species (Lovisolo y Schubert 1998, Schultz 2003). Thus, in this case, the reduction in g_s corresponded with a decay in K_{plant} in both cultivars and years (Fig. 1), confirming the positive relationship between g_s and K_{plant} (Pou et al. 2012; Martorell et al. 2015). This reduction in K_{plant} was explained by the hydraulic fatigue of xylem (Hacke et al. 2001), leading to reduce water availability and g_s in leaves. Declines in stomatal conductance have been hypothesized to respond more directly to K_{leaf} than K_{stem} (Tyree and Dixon 1986; Sperry and Hacke 2004; Bartlett et al. 2016). Thus, the driver of stomatal closure in Giró Ros could be related to K_{leaf} and $K_{petiole}$, revealing a high vulnerability to water stress, with 50 and 90% loss of conductivity, respectively (Fig. 3). This statement is in line with previous reports in grapevine and other species (Zufferey et al. 2011, Lauri et al. 2014).

In accordance with gas exchange and hydraulic conductance results, water withholding resulted in increased WUE_{leaf} (A_N/g_s, A_N/E and $\delta^{13}C$), similarly as has been previously described by several authors (Bota et al. 2001, Lovisolo et al. 2010, Tomas et al. 2012, Martorell et al. 2015). However, WUE_{wp} do not reflect those differences between treatments under those described experimental conditions. This lack of correlation between leaf WUE and WUE_{wp} has been described before in grapevines (Tomas et al. 2012). These differences could be explained by the complexity of processes determining WUE_{WP} like leaf water and carbon losses during the night and the stem and root respiration during the whole day, resulting in decreased WUE_{WP} while not changing (WUE_{leaf}) (Flexas et al. 2010, Tomas et al. 2012).

Virus infection

As expected, and in line with recent works studying the effect of GLRaV-1 and -3 (+) in Touriga Nacional (Moutinho-Pereira et al. 2012) and in Cabernet franc (Endeshaw et al. 2014), virus-infection (VI) did not affect Ψ_{Pd}. Our results showed that

Malvasia de Banyalbufar slightly developed an osmotic adjustment while Giró Ros did not show any change. It has been shown that certain virus concentrations may affect the carbohydrate transport which provides energy and solutes for osmotic adjustment (Shalitin and Wolf 2000, Gil et al. 2011, Fu 2010). Thus, as we have previously showed that Malvasia de Banyalbufar had a significant higher virus titre than Giró Ros (El Aou-ouad et al. 2016), we suggest that higher virus concentration results in an osmotic adjustment only in the former cultivar.

In addition, the presence of virus significantly decreased A_N, g_s and E, in both cultivars under WW conditions (Table 2). Accordingly, Endeshaw et al. (2014) showed that an active virus multiplication in the phloem affect stomatal closure independently of whole grapevine water status, thus being the presence of virus important enough to induce significant changes in the total plant water use, regardless of their water status. Additionally, our results were corroborated by previous works experiencing the same viral infection or other ones (Cabaleiro et al. 1999, Sampol et al. 2003, Bertamini et al. 2004, Moutinho-Pereira et al. 2012, Endeshaw et al. 2012, Grimmer et al. 2012) and within the same cultivars (Montero et al. 2016, El Aou-ouad et al. 2016). Despite VI decreased g_s, the studied cultivars showed unchanged hormone profiles. Contrarily, Whenham et al. (1986) observed that tobacco mosaic virus (TMV) infection dramatically increases ABA concentration in tobacco plants, resulting in g_s reduction. Thus, more studies are needed for better understanding the role of plant hormone in the regulation of g_s in response to virus infection.

In this study, we find a correlation of g_s with K_{plant}, K_{leaf} and $K_{petiole}$, which significantly decreased in VI plants. The slightly different effect observed on K_{plant} and K_{leaf} in Giró Ros VI plants, suggests different susceptibility of both cultivars to virus infection. Contrarily, recent research showed how plants infected with *Grapevine rupestris stem pitting associated* virus (GRSPaV) presented a decrease in specific root and shoot hydraulic resistance ($R_{hroot+shoot}$) (Pantaleo et al. 2016). Thus additional work needs to be done in order to evaluate if a general response to viral infection is affecting plant hydraulic parameters, or if it is virus-specific (Xu et al. 2008).

Remarkably, the presence of virus decreased more $K_{petiole}$ than $K_{leaf,}$ either in Malvasia de Banyalbufar and Giró Ros, revealing $K_{petiole}$ as an important parameter to be evaluated when studying the effect of virus on leaf hydraulic. This effect could be attributed to higher virus concentration in petioles than in leaves as previously reported Ling et al. (2001). Generally, Malvasia de Banyalbufar has been shown to be more affected than Giró Ros and consequently is probably due to the higher amount of virus found in the former cv. (El Aou-ouad et al. 2016).

In this work it is interesting to see that GLRaV-3 did not affect WUE_{leaf}, probably due to parallel reductions in g_s, A_N and E, (Table 2). This result is supported by unchanged leaf $\delta^{13}C$. In contrast, other studies in grapevine have shown that A_N/g_s and A_N/E were higher in GLRaV-1&-3(+) leaves and in infected plants with *Uncinula necator* (Powdery Mildew), respectively (Moutinho-Pereira et al. 2012, Bertamini et al. 2004; Lakso et al. 1981). In those cases, the reductions in g_s and E were more evident than the reduction in A_N.

WUE_{WP} exhibit the same tendency as WUE_{leaf}. Thus, despite the fact that virus infection provokes a loss of carbon assimilation, we might suggest that there was a compensation with reductions in respiration processes (Montero et al., 2016), thus resulting in an unchanged carbon balance.

Combination of water stress and virus infection

In our study, the combination of both stresses did not induce the expected additive effect on plant physiological and hydraulic parameters. Contrarily, other studies have described that the presence of WS can reduce or enhance the susceptibility of plants disease, and vice versa (Ramegowda and Senthil-Kumar., 2015). Xu et al. (2008) showed that an increase in relative water content (RWC) improved drought tolerance in plants infected with different virus such as Brome mosaic virus (BMV), Cucumber mosaic virus (CMV), Tobacco mosaic virus and Tobacco rattle virus. Moreover, other authors described an improvement in drought tolerance by GRSPaV (Pantaleo et al. 2016), even though, in this case the imposition of soil water stress was stronger than ours.

Remarkably, the combined stress reduced all the physiological parameters studied (A_N, g_s, E and Kh_{plant}) as compared to WW-NI plants (Table 2), whereas the interaction between virus infection and water stress (WS-VI) was not significant, indicating that both treatments affect in a different magnitude to those parameters, and that the combination of both did not reflect the expected additive effect. This could be explained by the high water stress effect over virus infection.

Regarding the hormonal response, the high ABA level in Malvasia de Banyalbufar could be explained by its possible role in pre-invasive defence (Ramegowda and Senthil-Kumar., 2015) forcing the plant to close stomata. Nevertheless, in the case of Giró Ros, ABA content did not play any role in stomatal closure. Thus, we may hypothesize that the predominant mode of drought response was, in this case, water potential-dependent stomatal closure as previously reported Brodribb et al. (2013), whereas in Malvasia de Banyalbufar, ABA could play an added role in stomatal defense response. Other studies also reveal a lack of relationship between the presence of ABA and g_s in different plants, including grapevine (Furukawa et al. 1990, Braatne et al. 1992, Brodribb et al. 2013, Tombesi et al. 2015).

K_{leaf} and $K_{petiole}$ were also affected by the combination of both stresses. Similarly, Choat et al. (2009) showed that WS plants infected with the bacteria *Xylella fastidiosa* (Xf) had greatly reduced K_{leaf}, suggesting that WS increases the vulnerability to this pathogen. They explained the decrease in hydraulic conductance by the possibility of having drought-induced embolism, vessel occlusions (gums, tyloses) or differences in xylem structure, however, in our case, GLRaV-3 is transported throughout the phloem (Maree et al. 2013) and thus, flow disturbance needs to be better understood.

Either at leaf and whole plant levels, the combination of stresses (WS-VI) had a pronounced effect on WUE. However, any interaction between stresses was observed in most of the parameters studied, the absence of further effect is probably due the dominant effect of water withholding over virus infection. Except for leaf $\delta^{13}C$, the interaction of both stresses was significant during the first year. Contrarily, in grapevine during ripeness, when water stress was present, Moutinho-pererira et al. (2012) showed that the presence of the virus induces an increase in A_N/g_s. From our results we can

conclude that the presence of virus in both cultivars did not increase water efficiency under water stress conditions.

CONCLUSION

Under WW conditions, there is a clear effect of the presence of virus on $K_{petiole}$. Thus, decreases of g_s in VI plants could be explained by the pronounced effect of virus in water flow. This is an important result to take into account for future works studying the effect of virus in leaf hydraulics. The current results revealed that K_{plant} was also affected by the presence of virus in Malvasia de Banyalbufar and Giró Ros in 2014.
Remarkably, the combination of stresses showed the same pattern of response that the one obtained for WS in terms of plant hydraulics and gas exchange. Although the presence of virus under water stress did not reflect any interactive response, this does not mean that virus infection is not affecting the plant physiology. Indeed, the effect of virus may be overshadowed by the presence of water stress.

Regarding WUE, our results show that WS was the only treatment significantly increasing WUE_{WP} and WUE_{leaf}. However, in virus infected plants, these parameters remained unchanged. We can conclude that in one-year old Malvasia de Banyalbufar and Giró-Ros VI plants, the presence of virus strongly affects plant physiology at the leaf level but not at the whole-plant level, where other factors such as respiration may help to obtain a positive carbon balance during virus infection.

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Supplementary table S1 Climatic conditions measured during growth and experimental periods in 2013 and 2014. Values represented are mean temperature (T◦ mean), Maximum temperature (T◦ max), evapotranspiration accumulated (ETPaccum) and vapour pressure deficit (VPD) per month. Different letters denote statistically significant differences among months in both experimental years at $P<0.05$ according to Duncan's test.

		Mean T (ºC)	Max T (ºC)	ETPaccum(mm)	VPD (KPa)
2013	**May**	16.5 ± 0.3^{a}	21.3 ± 0.4^{a}	118.5	0.66 ± 0.03^{a}
	June	21.3 ± 0.3^{b}	27.0 ± 0.5^{b}	146.5	1.09 ± 0.05^{b}
	July	26.1 ± 0.2^{c}	32.4 ± 0.4^{c}	168.2	1.51 ± 0.05^{c}
	August	25.6 ± 0.3^{c}	31.2 ± 0.5^{c}	128.4	1.36 ± 0.06^{b}
2014	**May**	18.1 ± 0.2^{a}	25.0 ± 0.4^{a}	127.3	0.71 ± 0.03^{a}
	June	23.1 ± 0.3^{b}	30.4 ± 0.5^{b}	161.9	1.20 ± 0.07^{b}
	July	24.9 ± 0.2^{c}	31.4 ± 0.3^{c}	169.0	1.30 ± 0.05^{c}
	August	25.4 ± 0.3^{c}	31.6 ± 0.4^{c}	141.3	1.01 ± 0.04^{b}

GENERAL DISCUSSION

The results of this PhD thesis have been structured in four chapters (1, 2, 3 and 4), each one corresponding to one publication. This research studied some of the main factors that could interfere in the recuperation of local grapevine cultivars, especially with the performance of those cultivars in the actual viticulture– virus incidence and water stress- as well as the effects of its interaction on growth, physiology and primary metabolites of two white local cultivars, Malvasia de Banyalbufar and Giró Ros. The present chapter contains a general discussion compiling the most important results of this thesis.

Recent research in old local cultivars pointed out the potential of these cultivars for future viticulture. They are adapted to specific climate conditions and several works revealed their potential resistances to drought as well as different capacities for better water use (Tomas et al. 2012, 2014; Bota et al. 2015). Moreover, some of these minor cultivars showed high enological aptitude (Escalona et al. 2009, 2012; Cretazzo et al. 2013; Bota et al. 2013; García-Muñoz et al 2014), especially for the white ones (e.g. Moll and Giró Ros varieties). Despite the importance of local cultivars in the different winegrowing regions, the survey of the sanitary status of these cultivars has remained unexplored. Recently, for many European autochthonous varieties, including some from the Balearic Islands, it has been shown that virus incidence is very common and this situation interferes with wine quality and modify some biodiversity features of these areas (Cretazzo et al. 2013). In order to contribute to the conservation and use of these cultivars, the first objective of this thesis was to study the sanitary status of 33 minority cultivars from the Balearic Islands (Spain) currently conserved in the Germplasm Collection (GCPM) and also to highlight the prevalence of *Grapevine leafroll associated virus-3* (GLRaV-3) in this collection.

The results obtained in the chapter 1, revealed that the local cultivars in the Balearic Islands were highly infected with single (39. 68%) and mixed infection (52. 07 %) and only 8.25 % of the total plants were virus-free. Moreover, our results also highlight the high incidence of GLRaV-3 (80 %) in all the cultivars studied. This finding could be explained by the high GLRaV-3 multiplication efficiency as compared to other ampeloviruses (Velasco et al. 2014).

Under this scenario of virus infection, the use of virus free material is of great importance. This situation may consolidate the necessity of the application of selection programs for recovering local cultivars and obtaining plants suitable for authorization and certification. In this chapter, two sanitation protocols were used for the sanitation of

double and triple viruses' infections. Our results revealed the successful elimination of the most common virus (GLRaV-3) as well as GFLV in the minority cultivars Argamussa and Gorgollassa. The application of thermotherapy in combination with shoot tips culture has proved to be simple, rapid and very effective method in virus erradication; with the possibility to eradicate up to three viruses in grapevine. Remarkably, virus elimination using only the shoot tips culture was also effective to obtain free plants. Both procedures reduce the time needed to regenerate healthy plants, in comparison with other protocols and could be applied to other interesting grape cultivars.

The multiple infections detected, especially high GLRaV-3 incidence in local cultivars, and the water deficit scenario in the Meditarranean area during summer lead us to formulate the second general objective of this Thesis. The second objective was to analyse the effects of GLRaV-3 infection (WW-VI), water stress (WS-NI) and the interaction (WS-VI) of both on grapevine physiology and primary metabolism. Table 1 summarizes the effects of all treatments (WS-NI, WW-VI and WS-VI) on the most important parameters measured in this study (see chapters 2, 3 and 4). The direction of the represented arrows indicates the sense (i.e, up- or down- regulation) and intensity (number of arrows) of the response of each of the indicated parameters. Equal sign means a lack of variation between treated and non-treated plants.

Water stress clearly decreased plant growth in terms of total leaf area, shoot length and total biomass accumulation (Table 1; Chapter 2: El Aou-ouad et al. submitted) as it is demonstrated in previous studies (Schultz and Matthews 1988, Poni et al. 1993, Escalona et al. 2002, 2003, 2012, Medrano et al. 2003; Van Leeuwen et al. 2009, Tomas et al. 2012). The biomass reduction is mainly caused by reductions in carbon assimilation, which depends on the balance between photosynthesis (A_N) and respiration (Flexas et al. 2010). A_N showed significant reductions under moderate water stress in both cultivars (Table 1; Chapter 3: El Aou-ouad et al., 2016). It is well established that under mild to moderate water stress, A_N limitation is mainly driven by reductions in diffusional factors rather than biochemical limitations (Flexas et al. 2004, 2006, 2009; Galmés et al. 2007; Tomás et al. 2014). Effectively, the results of the present Thesis (chapter 3) confirm that decreases in g_s and g_m largely explained the A_N limitation,being g_s the major determinant of this limitation.

In the present thesis, biochemical limitations (20%) was less important as compared to diffusional factors (80%), but still limiting photosynthesis. Hence, moderate water stress markedly decreased J_{max} but only had a small effect on V_{cmax} (Table 1), as previously observed Galle et al. (2011) and also De Souza et al. (2005) in grapevine. Several reports demonstrated that V_{cmax} is mainly preserved under mild drought, whereas its reduction has prevailed under severe drought (Bota et al. 2004; Grassi and Magnani, 2005; Galmés et al. 2007; Gallé et al. 2009). V_{cmax} depicts the in situ amount and kinetic properties of Rubisco. The effect of water stress on Rubisco is still under debate. Some studies show not changing rates in Rubisco activity (Vapaavuori 1986; Pelloux et al. 2001) while others, revealed that Rubisco content and activity decreased under severe water stress. In our study, the amount of TSP and Rubisco content remained invariable under moderate water stress, which is in line with some previous works (Parry et al. 2002; Bota et al. 2004). These contradictory set of results could be mediated because Rubisco activity is described to be species-specific (Parry et al. 2002; Tezara et al. 2002; Bota et al. 2004; Perdomo 201).

Nowadays, the study of metabolites profiling is becoming an important tool to discern the metabolic response of grapevine to water stress (Cramer et al. 2013; Hochberg et al. 2013, 2015). Those metabolies has been shown to fulfill different roles such as photosynthesis regulation, osmotic adjustment, protection against photoinhibition, and scavenging of reactive oxygen species (Verslues and Juenger 2011; Obata and Ferni et al. 2012; Cramer et al. 2013; Hochberg et al. 2013, 2015). An accumulation of sugars including sucrose, glucose, and fructose, and amino acids including proline and threonine has been reported in response to water stress conditions (Rolland et al. 2006; Ramel et al. 2009; Krasensky and Jonak 2012). In this study, water stress resulted in accumulation of proline and threonine (Chapter 4: El Aou-ouad et al. submitted), as was previously observed in grapevine (Cramer et al. 2013; Hochberg et al. 2013). However, soluble sugars such as glucose and fructose were not accumulated in the leaves of none of the studied cultivars and even decreased in Malvasia de Banyalbufar (Chapter 2: El Aou-ouad et al. submitted). This response has been reported before in grapevine under mild water stress (Hochberg et al. 2013) and in other plants (Rizhsky et al. 2004; Prasch and Sonnewald 2013; Jin et al. 2016). These results suggest no effects of mild water stress on sugar transport and partitioning as reported before in grapevine (Bota et al. 2004).

In summary, the results of the present thesis indicates that moderate water stress predominantly affect diffusional limitation to A_N, concretely g_s. The regulation of g_s under water stress is related to different physiological parameters such as leaf water potential, osmotic adjustments, hydraulic conductivity, chemical signals like ABA and others (Farquhar & Sharkey 1982; Ball et al. 1987; Leuning 1990, 1995; Buckley 2005; Martorell et al. 2015). The contribution of each parameter to the observed changes in g_s differed greatly among different studies. A recent study revealed that the g_s decline may result from the loss of turgor pressure in guard cells or other factors such as, root-derived abscisic acid (ABA) signals (Rodriguez-Dominguez et al. 2016). Another study has suggested that in drought grapevines, g_s is primarily regulated by passive hydraulic and then by active (ABA-mediated) mechanisms (Tombesi et al. 2016). In our study, [ABA] was only significantly increased in Malvasia de Banyalbufar but not in Giro Ros (Table 1; Chapter 4: El Aou-ouad et al. 2017). Different behaviors among grapevine varieties in relation to [ABA], K_{plant} and g_s values were already described by Martorell et al. (2015) for Tempranillo and Grenache. Different levels of foliar ABA accumulation were suggested to underlie different stomatal behavior in grapevine displaying as a consequence, near-isohydric or anisohydric hydraulic strategies (Soar et al. 2006). However, recent studies determined that the incidence of hydraulic mechanisms is the first signal triggering stomatal closure, with ABA playing a secondary role in regulating gas exchange (Tombsi et al. 2016). The latter study as well as others (Zufferey et al. 2011; Pou et al. 2012; Martorell et al. 2015; Lauri et al. 2014) are in agreement with the results obtained in our study (Table 1; Chapter 4: El Aou-ouad et al. 2017), highlighting the relevant role of hydraulic conductance parameters (K_{plant}, K_{leaf} and $K_{petiole}$) in stomatal control specially in Giró Ros.

The effect of water stress on gas exchange parameters was reflected in significantly increased leaf water use efficiency (WUE_{leaf}), with some differences between both cultivars (Chapter 4: El Aou-ouad et al. 2017). Our results are in line with a recent study (Bota et al., 2015), in which Giró Ros showed slightly higher WUE_{leaf} than Malvasia de Banyalbufar. Nevertheless, under water stress conditions, any consistent correlation was found between WUE_{leaf} and WUE at whole plant level (WUE_{wp}) as the later seemed unaffected (Table 1; El Aou-ouad et al. 2017), similarly to previous works in grapevine (Tomás et al. 2012). The main limitation in the lack of correlation between WUE_{leaf} and WUE_{wp} could be associated to the carbon losses by

plant respiration at different parts. Associated to the carbon losses by plant respiration at different parts, representing roots up to 60-65% of the total carbon used (Escalona et al. 2012).

As a part of the second objective of this thesis, the effect of virus infection on grapevine physiology and metabolite profiling were also analyzed.

Different studies revealed that GLRaV-3 has a significant impact in grapevine physiology (Cabaleiro et al. 1999; Bertamini et al. 2004, 2005; Moutinho-Pereira et al. 2012; Endeshaw et al. 2014). Most studies with plant-viral diseases have been focused in symptomatic plants; with supposed high virus concentration. However, studies about the effect of GLRaV-3 in asymptomatic plants and/or measuring the real virus concentration and its effects on plant physiology are still sparse (Montero et al. 2016d). The results of the present thesis clearly revealed that GLRaV-3 infection decreased photosynthesis as has been previously observed in the same or different grapevine cultivars (Sampol et al. 2003; Moutinho-Pereira et al. 2012; Montero et al. 2016b, 2016c). Although GLRaV-3 was markedly inhibiting leaf photosynthesis, any significant effect on plant growth has been observed (Chapter 2: El Aou-ouad et al. submitted). The latter result is in line with a recent finding from our group of research (Montero et. 2016b). The apparent lack of correlation between changes in A_N and biomass accumulation may be explained by the adjustment of carbon losses by respiration of the different plant organs, which was observed to compensate for the lower carbon assimilation in the presence of virus (Montero et al. 2016b). However, our results showed no effect on R_{leaves} by virus infection under well-watered conditions. Hence, further studies are necessary to dissect the effect of virus in the respiration of different plant organs.

Virus concentration was highly correlated with A_N, stomatal (g_s) and mesophyll (g_m) conductance, thus, in this case, photosynthesis (A_N) impairment was also associated with diffusional limitations (Chapter 2 and 3). Stomatal conductance was the main factor contributing to the decrease of A_N in Malvasia de Banyalbufar (Table 1) as was previously observed in grapevine (Cabaleiro et al. 1999; Bertamini et al. 2004; Montero et al. 2017). However, A_N reductions in Giro Ros were closely related to decreases in g_m as pointed out in other studies (Sampol et al. 2003; Moutinho-Pereira et al. 2012).

From our knowledge, the present study highlight for the first time, the relationship between g_m and leaf anatomy in response to GLRaV-3 infection (Chapter 3). It has been shown that g_m is highly determined by leaf anatomy in several species, including grapevine (Evans et al. 2009; Tosens et al. 2012; Tomas et al. 2014). Remarkably, in the present Thesis, thicker leaves in infected plants have a lower proportion of mesophyll cell surface area exposed to intercellular air spaces per unit leaf surface area (Table 1; Chapter 3), making CO_2 diffusion more difficult and limiting photosynthesis (Moutinho-Pereira et al., 2012). Despite reductions of A_N caused by GLRaV-3 were mainly due to diffusional limitations, GLRaV-3 infection also caused some biochemical limitations. GLRaV-3 infection reduced the maximum carboxylation efficiency (V_{cmax}) and the maximum electron transport rate (J_{max}) (Table 1), as was previously described by Endeshaw et al. (2014). We suggest that the effect of WS-VI on V_{cmax} is due to Rubisco activity, since Rubisco content was unchangeable in response to WS-VI (Table 1; Chapter 3- El Aou-ouad et al. 2016).

Comparing metabolite profiling with physiological parameters is a non-trivial task, therefore the combined analysis of those parameters developed in the present thesis can provide new insights for a deeper understanding the effect of virus on leaf physiology. The accumulation of soluble sugars (sucrose, fructose, glucose) in leaves from virus infected plants is thought to be related to metabolic feedback inhibition of photosynthesis (Bolton, 2009; Lemoine et al. 2009). An accumulation of several soluble sugars in response to Tobacco rattle virus (TRV) was observed in Arabidopsis (Fernández-Calvino et al. 2014). However, the results of the present thesis (Chapter 2) demonstrate that the levels of soluble sugars (fructose, glucose), metabolites of TCA cycle (malate and 2-oxo-glutarate) and Threonate tended to decrease in GLRaV-3 infected plants. Then, under these virus infection conditions no metabolic feedback inhibition occurred. The observed reduction in soluble sugars might be a consequence of their utilization for starch synthesis (Kogovšek et al. 2015). Thus, such different results may be due to the fact that the level of metabolites can oscillate in correlation with virus concentration (Bazzini et al. 2011). Virus infection produces not only alteration in plant photosynthesis, but also in several metabolic pathways. Interestingly, the results of the present thesis revealed that the alterations of the primary metabolism are mainly driven by changes on respiratory metabolism associated to cellular

requirements for plant defense responses (Berger et al. 2007; Gutha et al. 2010; Vega et al. 2011; Rojas et al., 2014; Montero et al. 2016c).

On the other hand, the results obtained in this thesis also highlight the effect of virus on other parameters, namely hydraulic and water use efficiency (Table 1). To our knowledge, this is the first study providing results about the effect of GLRaV-3 on hydraulic conductivity at whole plant (K_{plant}), leaf (K_{leaf}) and petiole ($K_{petiole}$) levels. Moreover the implication of GLRaV-3 on WUE at leaf (WUE_{leaf}) and whole plant (WUE_{wp}) levels is here studied for the first time. The results of the present thesis revealed that there is a clear effect of the presence of virus on K_{leaf} and $K_{petiole}$, with $K_{petiole}$ being much more affected than K_{leaf} in both cultivars. Thus, the effect of virus on $K_{petiole}$ can provide new insights to understand the g_s response to virus-infection (Table 1; Chapter 4). Moreover, K_{plant} it is shown to be correlated with g_s responses in virus infected plants.

Decreases in leaf hydraulics by VI were not reflected in WUE_{leaf} (intrinsic WUE (A_N/g_s), neither in instantaneous WUE (A_N/E) nor carbon isotopic composition ($\delta^{13}C$) (Table 1; Chapter 4). In other experiment, A_N/g_s was higher in GLRaV-1&-3(+) infected grapevine plants (Moutinho-Pereira et al. 2012). In the latter study, the reduction in g_s was higher than the reduction in A_N. Nevertheless, in the present Thesis it is interesting to see that under WW conditions GLRaV-3 did not affect A_N/g_s neither A_N/E due to the same magnitude of reduction in g_s, E and A_N (Chapter 4). In this case, WUE_{WP} exhibit the same tendency as WUE_{leaf}.

It is important to notice that for most of the parameters studied, the effect of virus showed cultivar-specific differences, being Malvasia de Banyalbufar more affected than Giró Ros (Table 1). On the one hand, we suggested that the slightly differences observed between cultivars could be explained by the higher amount of virus found in Malvasia de Banyalbufar (Chapter 3; El Aou-ouad et al. 2016). On the other hand, some studies have shown that the effect of grapevine leafroll and the severity of symptoms can vary greatly with the sensitivity of cultivars (Akbas et al. 2009). The relative effect of virus on physiological and metabolite profiling, as well as the differences observed in both studied cultivars, makes our results a good reference for performing future studies with different virus concentrations and cultivars to confirm if virus effect is concentration dependent and if there is a cultivar-susceptibility difference.

As we already discussed, many works have described the effect of water deficit or GLRaV-3 on plant physiology. However, the interaction/combination of GLRaV-3, or other virus, with water stress has only recently emerged as a new subject of study to understand the grapevine-virus-environment interaction. Thus, to our knowledge, the results of the present thesis establish for the first time the interactive effect of GLRaV-3 and water stress on grapevine performance, including plant hydraulics, water use efficiency and metabolite profiling. Thus, dissecting the physiological and metabolomics responses under combined stress conditions, leads us to achieve a better understanding of the mechanisms used by grapevines to cope with virus and water stress.

Recently, different works in Arabidopsis, barley and others crops highlight that the combination of different stresses provoke an additive effect on plant physiological parameters (Prasad et al. 2011; Vile et al. 2012; Rollins et al. 2013, Prasch and Sonnewald 2013; Perdomo et al. 2014). The results of the present Thesis have demonstrated that the combination between virus infection and water stress (WS-VI) decreases most physiological parameters in both cultivars studied as compared with WW-NI (Table 1). Indeed, significant reduction in net photosynthesis (A_N) under WS-VI was due to diffusional (g_s and g_m) and biochemical limitation; with stronger impairment of biochemical parameters as compared to WW-NI, i.e. maximum carboxylation efficiency allowed by the rubisco (V_{cmax}) and the maximum electron transport rate (J_{max}) (Table 1). A quantitative photosynthesis limitation analysis by Grassi and Magnani (2005) revealed that the relative contribution of stomatal (SL), mesophyll (ML) and biochemical (BL) limitations increased under WS-VI. Several works already have showed that g_m reflects, to a large extent, leaf anatomical parameters such as leaf thickness, leaf density, shape and wall thickness (Evans et al. 1994; Terashima et al. 2011; Tosens et al. 2012; Tomás et al. 2013). Likewise, the observed decrease of g_m in WS-VI plants seems to be related to the virus effect on leaf anatomical parameters, resulting in increased D_L and reduced intercellular air spaces (Chapter 3; El Aou-ouad et al. 2016). This is in accordance with what was previously shown by Evans et al. (1994).

Table 1: Summary of the effects of water stress in non-infected plants (WS-NI), virus infection in well-watered plants (WW-VI) and its interaction with water stress (WS-VI) on the main parameters considered in the present Thesis in the white local grapevine cultivars Malvasia de Banyalbufar and Giró Ros.

Parameters		WS-NI		WW-VI		WS-VI	
		Malvasia	Giro-Ros	Malvasia	Giro-Ros	Malvasia	Giro-Ros
Growth Parameters	LA	↓↓	↓↓	↓=	=	↓↓	↓↓
	Shoot lenght	↓↓	↓↓	↓	=	↓↓	↓↓
	TBI	↓↓↓	↓↓↓	↓↓=	=	↓↓↓	↓↓↓
Physiological Parameters	A_N	↓↓	↓↓	↓↓	↓	↓↓	↓↓↓
	g_s	↓↓↓	↓↓↓	↓↓	=↓	↓↓↓	↓↓↓
	g_m	↓	↓↓	=	↓↓	↓	↓↓
	Kh_{plant}	↓↓	↓↓	↓↓	↓=	↓↓↓	↓↓↓
	K_{leaf}	-	↓↓	↓↓	=	-	↓↓↓
	$K_{petiole}$	-	↓↓↓	↓↓	↓↓↓	-	↓↓↓
	WUE_{wp}	=	=↑	=	=	=	=↑
	A_N/g_s	↑↑	↑↑	=	=	↑↑	↑↑
	V_{cmax}	=	=	↓↓	↓↓	↓↓	↓↓↓
	J_{max}	↓↓	↓↓	↓↓	=	↓↓↓	↓↓↓
Anatomical Parameters	T_L	=	↑↑	↑↑	↑	↓↓	↓↓
	M_A	=	↓	↑↑	=	=	↓↓
	D_L	=	↓↓	↑↑	=	↑↑	↑↑↑
	f_{ias}	↓↓	↓↓	=	↓↓	↓↓	↓↓↓
Hormonal Status	[ABA]	↑↑	=	=	=	↑↑↑	=
Metabolites level	Glucose	↓	=	↓	↓	↓↓	↓↓
	Proline	↑	↑	=	=	↑↑	↑↑
	Threonine	↑	↑	=	=	↑↑	↑↑

LA, leaf area; TBI, total biomass increment; A_N, net photosynthetic rates; g_s, stomatal conductance ;g_m, mesophyll conductance; Kh_{plant}, whole plant hydraulic conductance; K_{leaf}, leaf hydraulic conductivity; $K_{petiole}$, petiole hydraulic conductivity; WUEwp water use efficiency at whole plant level; A_N/gs, instantaneous water use efficiency; V_{cmax}, maximum carboxylation rate; J_{max}, maximum photosynthetic electron transport rate; T_L leaf thickness ; M_A leaf mass per area; D_L leaf density; f_{ias}, mesophyll porosity . Those parameters were explained in the chapters 2, 3 and 4.

Interestingly, our results showed that, although the combination of both stresses did not cause any additive effect in grapevine physiological parameters, most of the metabolic changes under WS-VI were specific and not quantitatively predicted from the sum of responses to each single stress (Chapter 2). Furthermore, it is interesting to note that the specificity of these metabolic responses under combined stress is also supported by significant correlation between metabolites and leaf respiration (R_{leaves}). In such correlations, it is observed that most of the metabolites correlating with R_{leaves} under WW-VI were different to those correlating with R_{leaves} under WS-VI (Chapter 2). This result strongly suggests that respiration can play a primordial role in the metabolic adjustment under combined stress conditions.

The effect of WS-VI was also reflected in water flow and water economy parameters (Table 1; Chapter 4).

It has been recently shown by several authors (Pantaleo et al., 2016; Xu et al., 2008; Ramegowdaa and Senthil-Kumar., 2015) that the presence of WS can reduce or enhance the susceptibility of plants to a biotic pathogen, and vice versa. In Pantaleo et al. (2016), GRSPaV-infected plants alter grapevine responses to drought by increasing the photosynthesis rate and the stomatal conductance, and by reducing hydraulic resistance to water transport as well as increasing the ability to extract water from the soil. Thus, in this case, a positive interaction between virus and water stress has been described. The results of the present Thesis revealed that WS-VI decreased plant hydraulic parameters (K_{plant}, K_{leaf} and $K_{petiole}$), having the most pronounced effect on petiole hydraulic conductivity (Chapter 4). Further research is required to fully understand the mechanisms underlying such effect of phloemetic virus on water flow.

Additionally, water use efficiency (WUE_{leaf} and WUE_{plant}) was also reduced by WS-VI as compared to WW-VI. However, the interactive effect of WS and VI was not additive as compared to single WS-NI, suggesting that the investigated stresses could exert an independent effect. The absence of further additive effects may be explained either by the higher effect of WS than VI or by the compatible interaction between virus and plants as argued by previous works (Gambino et al. (2012). On the other hand, some effect of WS on virus replication cannot be discarded. In this respect, the virus concentration in Malvasia de Banyalbufar cv. (2013) and in Giro Ros cv. (2014) was significantly ($P<0.05$) lower under WS than under WW conditions (Chapter 2).

In summary, the present Thesis highlight that the interaction between virus infection and water stress was not additive at physiological level in both cultivars, resulting in similar effect as single water stress (Table 1). Moreover, the response of grapevine to combined stress is proved to have specific response at metabolic level. Thus, these patterns should be taken into account in future works to better understand the grapevine-virus-interaction within climate change scenarios.

CONCLUSIONS

From the results and discussion of the present Thesis, a series of conclusions can be drawn with regard to the objectives established in the current Thesis.

1. To study the sanitary status of Majorcan minority grapevines cultivars and to highlight the prevalence of Grapevine Leafroll-associated virus 3 (GLRaV-3) in local cultivars

1. Analysis of sanitary status of Majorcan local grapevines cultivars demonstrated that those cultivars were highly infected with single and mixed virus infection.
2. GLRaV-3 was the most prevalent virus in the local cultivars (82%). Even though the incidence of GFkV and GFLV were also frequent (45% and 25%, respectively).
3. Two sanitation techniques "shoot tips culture and thermotherapy in combination with shoot tips culture" have been optimized for double and triple viruses' eradication in two local cultivars, Argamussa and Gorgollassa.

1. *To determine the effects of water deficit, GLRaV-3 infection and its combination on plant growth and primary metabolism*

And

2. *To determine the effects of water deficit, GLRaV-3 infection and its combination on photosynthesis identifying which part of the photosynthetic machinery was mainly affected (diffusion or biochemical limitations)*

4. Moderate water stress decreased total plant growth that was associated with photosynthesis limitations, mainly due to reduced diffusional factors (gs and gm).
5. Moderate water stress resulted in some changes in primary metabolism as proline and threonine accumulation; however no sugar accumulation was observed in leaves, reflecting no effects on sugar transport nor feedback inhibition of photosynthesis.
6. Under WW conditions, GLRaV-3 significantly reduced photosynthesis, mainly associated with diffusional limitations, althought in general this was not reflected on growth parameters. Moreover, a good correlation

between metabolites changes and respiration suggest an active metabolic adjustment of carbon losses.

7. GLRaV-3 infection also caused some biochemical limitations, reducing maximum carboxylation efficiency (V_{cmax}) and maximum electron transport rate (J_{max}).
8. The combination between virus infection and water stress decreases most physiological parameters in both cultivars, however, did not further increase the effects on plant growth or leaf gas exchange parameters, as compared to single water stress.
9. At metabolic level, responses to combined stress were specific and not quantitatively predicted from the sum of responses to each single stress. The specific adjustment of respiratory metabolism can explain the maintenance of leaf carbon balance and growth in grapevines under combined stress conditions.

3. To investigate the effect of water deficit, GLRaV-3 infection and the combination of both stresses on on hydraulic conductance and consequences on water use efficiency at leaf and plant levels

10. Water stress increases leaf WUE (A_N/g_s; A_N/E and $\delta^{13}C$), but not whole plant WUE. Water withholding was also reflected in decreased hydraulic parameters ($K_{petiole}$, K_{leaf} and K_{plant}), associated with a tight stomatal control.
11. GLRaV-3 infection did not affect leaf and whole plant WUE under well watered nor water stress conditions. Nevertheless, a significant correlation between g_s and hydraulic conductances ($K_{petiole}$, K_{leaf} and K_{plant}) in response to GLRaV-3 infection suggest a tight regulation in water flow in the presence of virus. The stronger effect of virus on $K_{petiole}$ than in K_{leaf} and K_{plant} is an important key to tack into account in future works.
12. Combined stress do not increase the effects on WUE and hydraulic conductance parameters; the presence of virus did not increase water efficiency under water stress conditions.

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