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Assessing the Influence of Viral Infection on 'Tribidrag' Grapevines: Insights from Two Vegetation Seasons

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Abstract: The objective of this study was to investigate the response of the grapevine variety 'Tribidrag' to virus infection over two vegetation seasons. Virus-free plants were greenhouse cultivated and green grafted with five different virus inocula composed of grapevine leafroll-associated virus 3 (GLRaV-3) singly or in coinfection with other most economically important grapevine viruses. Changes in nutrient status and photosynthesis-related parameters, along with symptom development, were measured. Using the quantitative PCR method, the relative concentration of five selected *Vitis* genes was determined. Cluster analysis and ANOVA revealed the reduction in phosphorus concentration (P) and photosynthesis-related parameters in infected plants in both seasons, even in the absence of symptom expression, indicating P and assimilation rate (Photo (A)) as stable markers of virus infection. Plants infected with inoculum Y composed of five different viruses provoked major significant changes in the first season while, in the second, fewer changes were measured. The sucrose synthase 3 gene was upregulated in infected plants confirming disturbed sugar metabolism related to virus-induced stress. This study showed that virus-induced changes in 'Tribidrag' plants even in the absence of symptoms are dependent on plant age, as well as on the composition of virus inocula.

Keywords: grapevine; grapevine viruses; 'Tribidrag'; phosphorus; photosynthesis

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1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the most important perennial crops worldwide that is affected by the largest number of viruses and virus-like agents [1]. Several diseases of viral etiology are so far recognized as economically important such as grapevine leafroll disease (GLD), infective degeneration, fleck disease, and rugose wood (RW) disease [2] with a new one emerging in European continent known as grapevine leaf mottling and deformation syndrome (GLMD) [3]. Grapevine viruses are spread over long distances by using infected propagation material and locally through vector transmission, mainly nematodes, mealybugs, and scale insects [4]. The infected plants in the field can be asymptomatic [5], which can further facilitate the spread of viral diseases by making it difficult to identify and remove the infected source plants. All the mentioned viral diseases diminish the grapevine performance while having a profound impact on its physiological processes, reducing its production lifespan and crop quality [2]. The disease impact on the grapevine host plant is not unvarying as it can be under the strong influence of multiple factors such as coinfecting viruses, environmental conditions, vegetation season, grapevine variety, and a specific rootstock-scion combination [6].

In several studies conducted so far on the occurrence of economically important viruses in indigenous varieties of the Croatian Mediterranean region, grapevine leafroll-associated virus 3 (GLRaV-3), the main causal agent of GLD [7], has been singled out as

the most prevalent virus [5,8,9]. Due to the known negative impacts of GLD on grapevine physiology, such as reduced photosynthesis [10–12], reduced yield and fruit quality [13,14], and disturbed oxidative balance [15], the qualitative potential of indigenous varieties is limited, making them less attractive to grapevine producers [16]. Furthermore, the influence of GLD on changes in the grapevine transcriptome is described in leaves, where upregulation of flavonoid biosynthetic pathway genes occurs as a consequence of the accumulation of soluble sugars [17]. The production of signaling molecules such as salicylic acid has also been reported as a direct consequence of GLD in grapevine [17]. GLRaV-3 in indigenous varieties of the Croatian Mediterranean region is almost exclusively detected in mixed infections, most often with causal agents of rugose wood disease [5,8,16,18], and in most recent studies, with grapevine pinot gris virus (GPGV) [5,8], the main causal agent of GLMD. This deteriorating sanitary status of indigenous varieties emphasizes the need for using virus-free planting material in order to achieve the full potential of Croatian indigenous varieties [5].

Out of the Croatian indigenous varieties, 'Tribidrag' stands out as an economically important one, regaining more popularity with Croatian winemakers [19] due to its qualitative potential for producing premium red and dessert wines [20]. Widely grown in Italy by the synonym Primitivo and in the USA by the synonym Zinfandel [19], it is genetically related to many Croatian indigenous varieties, including the parentage of our most widespread red variety 'Plavac Mali' [21]. This, along with many historical mentions of 'Tribidrag' in the Mediterranean part of Croatia [19], proves that this was one of the most important varieties in this wine-growing area. Due to its higher susceptibility to fungal diseases it was almost completely abandoned in the late 19th century upon the arrival of phylloxera and cryptogamic diseases, but now is regaining momentum in Croatian viticulture, with a growing number of new vineyards in the Mediterranean part of Croatia [20]. With this in mind, in this study, we characterized the response of the indigenous variety 'Tribidrag' to the most important grapevine viruses previously detected in indigenous varieties of the Mediterranean part of Croatia. Our aims were to evaluate the susceptibility of 'Tribidrag' to different viruses or combinations of viruses over a period of two vegetation seasons and to measure the parameters reflecting plant fitness even in the absence of visual symptoms. On own-rooted plants in greenhouse conditions, we observed changes in physiological, morphological, and transcriptomic responses to virus infection.

2. Materials and Methods

2.1. Greenhouse Experiment Setup

The one-year-old cuttings of cv. 'Tribidrag' used in this study were taken from the virus-free vineyard of the Institute for Adriatic Crops in Split, Croatia. Planting material for virus-free vineyard establishment was previously sanitized from all known viral and other pathogens at the Foundation Plant Service (University of California, Davis, CA, USA). 'Tribidrag' cuttings were rehydrated, treated with fungicides, and immersed briefly in the 2000 µg/mL indole-3-butyric acid solution (IBA, Sigma Aldrich, Darmstad, Germany). After immersion, cuttings were planted in the mixture of perlite and peat in a 3:1 ratio and regularly irrigated to prevent root drying out. Four weeks later, plants that successfully developed roots were replanted in 6 L pots that contained a mixture of brown soil:peat (Brill type 5, Kekkilä-BVB, Georgsdorf, Germany):perlite (Agrilit 3, Perlite espansa; Perlite Italiana, Milano, Italy) in 1:1:1 ration and 1/3 of volume of quartz sand (Lasselberger–Knauf, Đurđevac, Croatia) was added in the mixture. Plants were grown in an experimental greenhouse (Schwarzman) under natural light, and during the vegetation period, the temperature ranged from 18 to 35 °C. Plants were irrigated with ¼ of Hoagland solution [22] once a week at the beginning of vegetation and once every two weeks when plants reached full growth. Hoagland solution was administered to provide all the macro- and micronutrients essential for grapevine growth and development.

In the summer of the second vegetation year, plants were inoculated using the green grafting technique [23] with five different inocula containing GLRaV-3 singly or in combi-

nation with other viruses (Table 1). The specific combinations of viruses were identified in prior research by Hančević et al. [8], who found them to be the most prevalent among indigenous grapevine varieties in Mediterranean Croatia. Post-inoculation grafted plants were intensively watered and a single use of ammonium nitrate fertilizer (1 g/L, Petrokemija, Kutina, Croatia) was applied to induce new vegetative growth.

Table 1. Virus composition of inocula used for infecting 'Tribidrag' plants.

Inoculum	Virus Composition
II	GLRaV-3 *
X	GLRaV-3, GVA, GRSPaV, GPGV
Y	GLRaV-3, GLRaV-1, GVA, GRSPaV, GPGV
Q	GLRaV-3, GLRaV-2, GVA, GFkV, GRSPaV, GPGV
Z	GLRaV-3, GVA

* Abbreviated names for individual viruses stand as listed: GLRaV-3—grapevine leafroll-associated virus 3, GVA—grapevine virus A, GPGV—grapevine pinot gris virus, GRSPaV—grapevine rupestris stem pitting associated virus, GLRaV-1—grapevine leafroll-associated virus 1, GLRaV-2—grapevine leafroll-associated virus 2, GFkV—grapevine fleck virus.

2.2. Virus Transmission Confirmation and RNA Extraction Procedure

Virus transmission was first confirmed three months post-inoculation by DAS-ELISA [24], which was performed on petiole samples using the commercially available kit (Agritest, Valenzano, Italy) specifically targeting GLRaV-3. Two hours after adding substrate p-nitrophenylphosphate buffer, the absorbance was recorded at 405 nm, and values 3 times greater than the mean absorbance value of the negative control were considered positive for virus presence.

Final confirmation of successful virus transmission was obtained by using multiplex RT-PCR as described by Gambino and Grimaudo [25], for the following viruses: GLRaV-1, GLRaV-2, GLRaV-3, grapevine fanleaf virus (GFLV), arabis mosaic virus (ArMV), GFkV, GVA, grapevine virus B (GVB) and GRSPaV. Transmission of GPGV was confirmed in a separate RT-PCR reaction as described by Saldarelli et al. [26].

For multiplex, RT-PCR RNA was extracted from cortical scrapings of each individual infected plant and control plants using the Rapid CTAB method, as described by Gambino et al. [27]. RNA extracts were purified from any remaining DNA using the TURBO DNA-free™ Kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Reverse transcription was performed using MMLV reverse transcriptase (Invitrogen, Waltham, MA, USA) with the addition of 100 units of RNase inhibitor (Invitrogen, Waltham, MA, USA) and 5 µM random nonamers (Sigma Aldrich, St. Louis, MO, USA) with 500 ng of RNA template.

2.3. Gene Expression Analysis

For this study, five genes of interest were chosen from the flavonoid biosynthetic pathway, salicylic acid signaling pathway, and sugar metabolism-related enzymes (Table 2). These genes of interest are chosen to reflect particular physiological processes in grapevines that are known to be impacted by virus infection, as proven with other grapevine varieties [17]. From mature leaf samples of each individual plant, RNA was extracted using the Rapid CTAB method by Gambino et al. [27] in three biological replicates per treatment. Leaves were sampled in the second year of measurements, 25 months post-inoculation. The RT-qPCR mix contained 1×Sybr green (Bio-Rad, Hercules, CA, USA), primer concentrations as listed in Table 2, and 0.5 µL of cDNA, in a total reaction volume of 10 µL. Cycler conditions used were 95 °C for 30 s, followed by 45 cycles of denaturation at 94 °C for 10 s and an extension step at 60 °C for 30 s. Melt curve analysis was also performed to verify the specificity of the reaction.

The relative concentration of target genes (ΔCt) was calculated by subtracting their Ct values from those of the geometric mean of two reference genes (actin and α-tubulin) [28].

Table 2. Primers used in gene expression analysis of 'Tribidrag' plants.

Primer Name	*V. vinifera* Genomic Region	Sequence (5′-3′)	Concentration in qPCR Mix	Reference
VV_actin_F VV_actin_R	Actin	F: CTTGCATCCCTCAGCACCTT R: TCCTGTGGACAATGGATGGA	0.4 µM	Reid et al. [29]
VV_tubulin_F VV_tubulin_R	Tubulin	F: CAGCCAGATCTTCACGAGCTT R: GTTCTCGCGCATTGACCATA	0.4 µM	
LAR2_F LAR2_R	Leucoanthocyanidin reductase 2	F: TGATATCAGCTGTGGGTGGA R: CCCAAATTCTGATGGAAGGA	0.48 µM	Gutha et al. [30]
F3H2_F F3H2_R	Flavanone 3-dioxygenase	F: CTGTGGTGAACTCCGACTGC R: CAAATGTTATGGGCTCCTCC	0.48 µM	
NPR1_F NPR1_R	Structural domain containing NPR1 protein	F: GTGGCGGTTTTGGGGTATTTGT R: GCACCTCCACCATGAAATCCAC	0.33 µM	Orrantia-Araujo et al. [31]
SPS_F SPS_R	Sucrose-phosphate synthase	F: CAGGGTCGACCTCTTCACTC R: ATATGGCCAAACAGGCTGAC	0.4 µM	Ren et al. [32]
SS3_F SS3_R	Sucrose synthase 3	F: GCCCTGCATGGTTCAATTGA R: GTCAAGCCTTGCCATGGAAA	0.4 µM	

2.4. Sample Collection

Leaves were sampled 13 and 25 months post-inoculation, during two successive vegetational seasons, starting from the second year after inoculation. Samples were taken from all plants from individual treatments and treated as biological replicates (n = 5). The first year was omitted from sampling due to the short period of vegetation in the post-inoculation period. Samples for determining nutrients and photosynthetic pigment concentrations were subjected to freeze-drying before being pulverized into fine powder which was used in the analysis.

2.5. Elemental Composition of Leaves

For nutrient analysis, 0.5 g of lyophilized leaf samples were dried at 550 °C for 5 h, after which 2 mL of HCl was added before the final dilution of samples to the volume of 50 mL with ddH20. Phosphorus concentrations were determined by the method of Olsen et al. [33]. Potassium concentration was determined using a flame photometer (Model 410, Sherwood, Cambridge, UK), and concentrations of iron, zinc, manganese, copper, calcium, and magnesium were measured by atomic absorption spectrometer (SpectrAA 220, Varian, Palo Alto, CA, USA).

2.6. Physiological and Morphological Analysis in Plants

Photosynthetic pigment concentrations (Chlorophyll a, chlorophyll b, total carotenoids) were measured by the method described by Lichtenthaler [34], relative water content was measured by the method described by Gucci et al. [35] and membrane permeability as described by Tarhanen et al. [36].

From morphological parameters, the average internode length was determined by dividing the length of an individual cane by the number of buds on that same cane.

Gas exchange measurements were performed using Li-COR 6400 (LI-Cor Inc., Lincoln, NA, USA), calibrated as follows: inner CO_2 concentration 400 ppm, light intensity 500 µmol m^{-2} s^{-1}, 90:10 ratio of red and blue light, relative air humidity 50%, and block temperature 25 °C. Parameters measured were leaf transpiration intensity (Trmmol), assimilation rate (Photo (A)), stomatal conductance (Cond(gs)), intercellular CO_2 concentration (Ci), and quantum yield from CO_2 assimilation (PhiCO_2). All measurements were performed between 10:00 and 13:00 h.

2.7. Symptom Evaluation

Symptom expression of viral diseases: grapevine leafroll disease (GLD), rugose wood (RW), grapevine leaf mottling and deformation (GLMD), and grapevine fleck disease were assessed throughout the two consecutive vegetation seasons. Appearance of leaf reddening

and downrolling associated with GLD, leaf mottling and deformation associated with GLMD, malformations on the woody part of the plant associated with RW disease, and leaf flecking, and upward curling for fleck disease were assessed. Individual plants were classified as symptomatic or asymptomatic.

2.8. Statistical Analysis

All statistical tests were performed in R (v4.3.2). To observe the specific effects of the individual inoculum on 'Tribidrag' plants, results from physiological and morphological parameters were compared with virus-free control plants using the ANOVA test with the post hoc Tukey test. The pheatmap package (v1.0.12) was used to generate heat maps and to conduct cluster analysis [37] and for conducting non-parametric tests for gene expression data the dunn.test package (v1.3.5) was used [38].

3. Results

3.1. Virus Transmission Confirmation

All of the green grafted plants tested positive for GLRaV-3 by ELISA on petiole samples obtained three months post-inoculation. Following the ELISA results, we could assess the successful virus inoculation by grafting (Supplementary Table S1). Transmission of all other viruses, which constituted individual inoculums, was confirmed by RT-PCR (Supplementary Figure S1).

3.2. Results of Physiological and Morphological Analysis in Plants

The heat map generated from 21 parameters measured in the first year revealed two major clusters with control plants clustered with plants infected with II and Y inocula. Plants infected with Q, X, and Z inocula were grouped in a separate cluster (Figure 1). All infected plants were characterized by lover content of Mn, Ca, P, Mg, along with photosynthetic parameters (Photo, PhiCO_2, Cond, and Trmmol) and relative water content (RWC) compared to control plants. Plants infected with inoculum Y were separated from the control plants and other infected plants due to having lower concentrations of photosynthetic pigments along with lower concentrations of K_2O, Cu, and Fe. The same plants had a higher concentration of Zn and higher dry weight of the leaves (DW leaves). In the second year, the heat map that was generated with the same parameters revealed fewer differences, except in the case of P concentration, Photo, and A/Ci, where control plants separated from all infected plants (Supplementary Figure S2).

In the first year of measuring, we observed a higher number of significantly altered parameters when compared with the second year (11 versus four, Figures 2 and 3, Supplementary Figures S3 and S4), and a higher number of altered parameters were related to the nutrient status of the 'Tribidrag' plants, especially, in the case of those infected with inoculum Y (Figure 2). Significant changes in the second year were related to photosynthesis-related parameters and phosphorus concentration (Figure 3).

Symptom expression of viral diseases was almost completely absent in the two vegetation seasons in which they were observed. The only exceptions were symptoms of grapevine leafroll disease (GLD), which were noted in both vegetation seasons in 'Tribidrag' plants infected with Y inoculum and also in 'Tribidrag' plants infected with Q inoculum in the second vegetation season. Symptom development of GLD is listed in Supplementary Table S3.

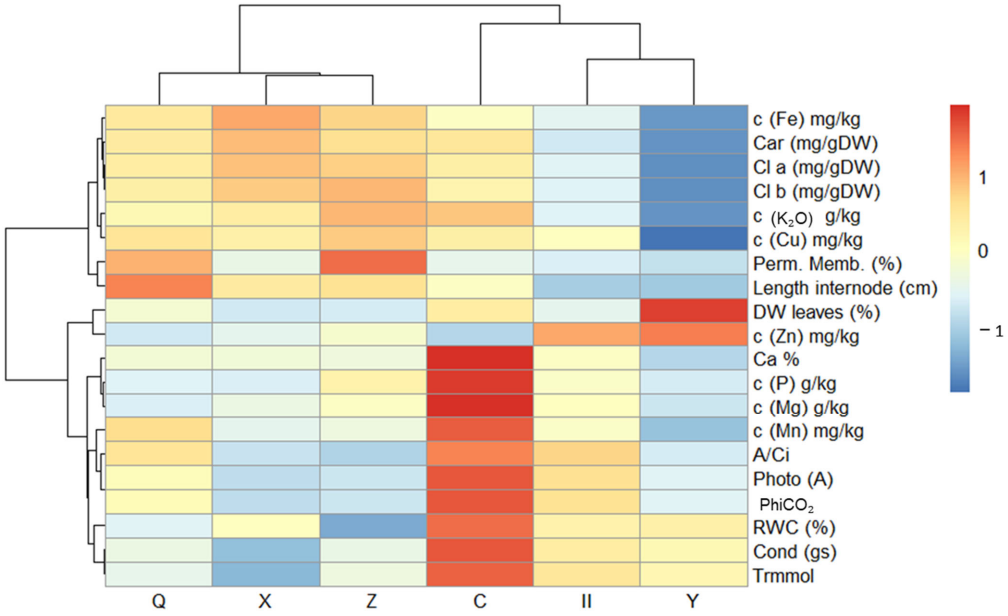

Figure 1. Heat map analysis calculated from measured parameters in 'Tribidrag' plants infected with GLRaV-3 singly or in coinfection with other viruses (Table 1) in the first year of measurements, control plants are marked with C on the *x*-axis. Distance was determined by Euclidean method (columns) and clustering (rows) was performed using Ward method [39]. Abbreviations are as follows: c—concentration, Fe—iron, Car—Carotenoids, Cl a and b—Chlorophyll a and b, K_2O—Potassium, Cu—Copper, Perm. Memb—Membrane permeability, DW leaves—dried weight of leaves, Zn—zinc, Ca—calcium, P—phosphorus, Mg—Magnesium, Mn—manganese, Photo (A)—assimilation rate, Cond (gs)—stomatal conductance, $PhiCO_2$—Quantum yield from CO_2 assimilation, Trmmol—leaf transpiration, RWC—relative water content.

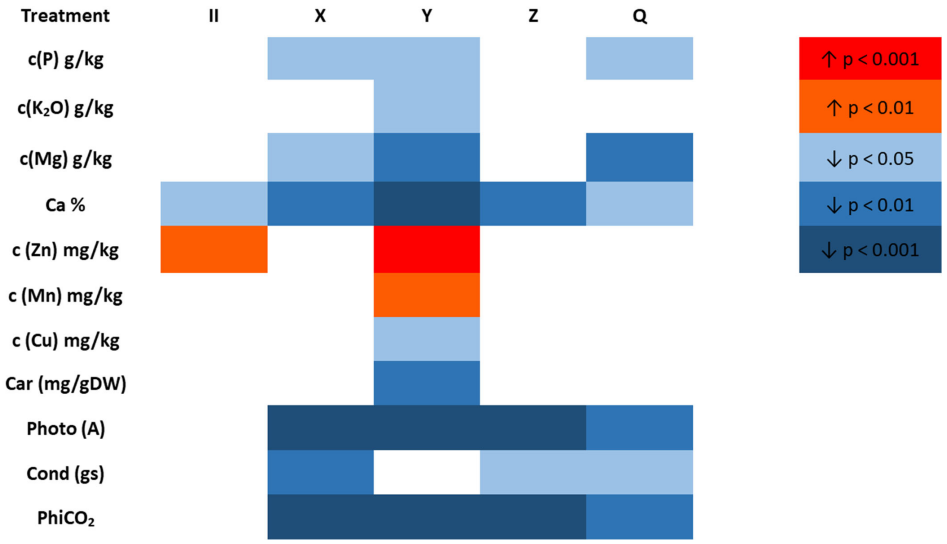

Figure 2. Results of ANOVA test and post hoc Tukey comparing the changes in measured parameters between control and infected plants in the first year of measurements. Significant changes are marked as follows: $p < 0.05$, $p < 0.01$, and $p < 0.001$, where the color coding and arrows direction on the legend reflects the direction of changes, with shades of blue indicating a significantly lower content (the arrow in down direction) and shades of red significantly higher content (the arrow in up direction). Abbreviations for individual parameters are as follows: c—concentration, P—phosphorus, K_2O—potassium, Mg—magnesium, Ca—calcium, Zn—zinc, Mn—manganese, Cu—copper, Car—total carotenoid content, Photo (A)—assimilation rate, Cond (gs)—stomatal conductance, $PhiCO_2$—Quantum yield from CO_2 assimilation.

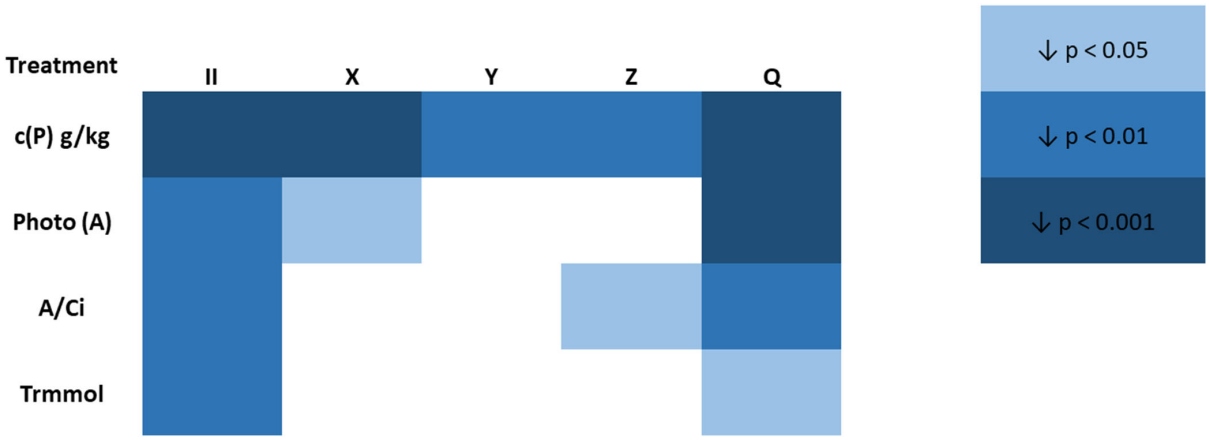

Figure 3. Results of ANOVA test and post hoc Tukey comparing changes in measured parameters in the infected with the control plants in the second year of measurements. Significant changes are marked as follows: $p < 0.05$, $p < 0.01$, and $p < 0.001$, with different shades of blue representing significantly lower content, also indicated with the arrow's down direction on the legend. Abbreviations for individual parameters are: c—concentration, P—phosphorus, Photo (A)—assimilation rate, Ci—Substomal CO_2 concentration, Trmmol—Leaf transpiration.

3.3. Gene Expression Analysis

Out of the five genes that were analyzed in terms of their relative expression, the sucrose synthase gene (SS3) was upregulated in the 'Tribidrag' infected plants inoculated with Q, Y, and Z inocula compared to the control plants (Figure 4). LAR2, F3H2, NPR, and SPS genomic regions did not show any significant differences in terms of their relative expression compared to control plants. Raw data obtained from the qPCR analysis of gene expression are available in Supplementary Table S4.

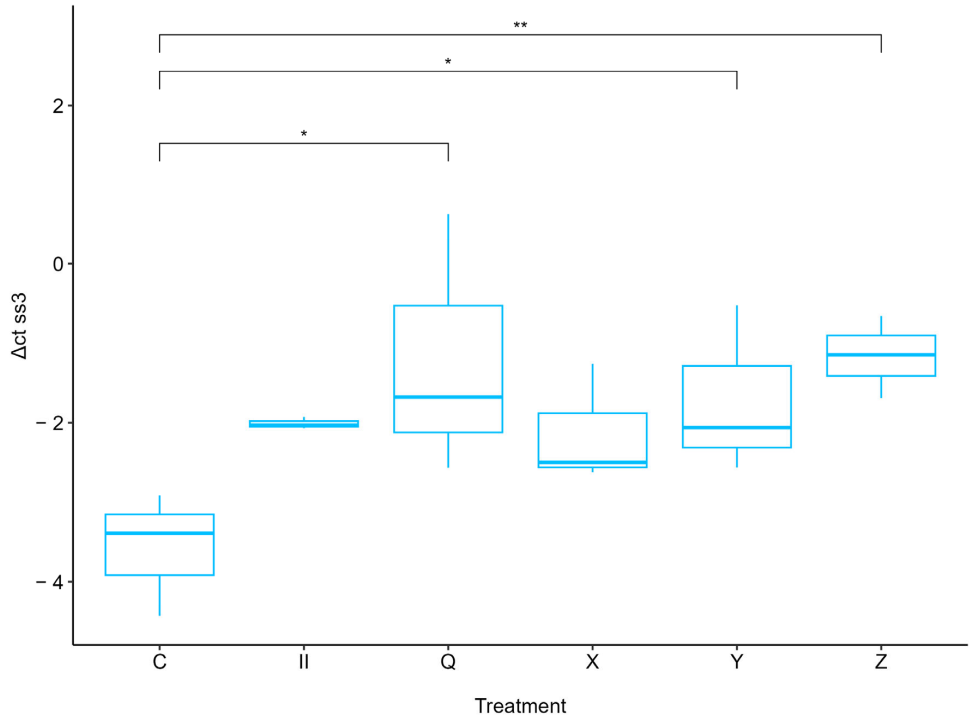

Figure 4. Relative concentration of sucrose synthase gene (Δct SS3) for 'Tribidrag' plants infected with different virus inocula. Non-parametric Kruskal–Wallis test was performed along with Dunn post hoc test and the significantly different values are indicated over individual boxplots ('*'—$p < 0.05$, '**'—$p < 0.01$). Abbreviations for individual treatments are listed in Table 1.

4. Discussion

In this study, the response of 'Tribidrag' to virus infection was proven to be time-dependent in terms of infection duration and plant age, as well as the composition of the virus inocula. Heterogenous responses to virus infection of 'Tribidrag' plants were proven by the differing clustering of most of the measured parameters over the two years (Figure 1 and Supplementary Figure S1), with fewer parameters being significantly altered in the second year as opposed to the first year (Figures 2 and 3). This trend is expected, as older plants are generally more tolerant to virus infection [40].

The parameters that were significantly impacted by virus infection in both years refer to phosphorus concentration and photosynthesis-related parameters, which were significantly lower when comparing infected to control plants (Figures 2 and 3). Reduced phosphorus concentration was previously reported only, to the best of our knowledge, in the case of coinfection of GLRaV-3 and GLRaV-1 [14]. In this study, we confirmed it for most of the virus inocula in the first year and for all inocula in the second experimental year. The macronutrient phosphorus is one of the most important elements in grapevine physiology with a significant role in photosynthesis either as an energy source, a regulator of the number of enzymes and signal receptors [41], or as a building block molecule of one of the most important plant enzymes—Rubisco [42]. The reduction in phosphorus concentration in infected plants in our study was accompanied by changes in the photosynthesis-related parameters, where, in both years, most of the infected plants had a significantly lower assimilation rate (Photo (A), Figures 2 and 3). In the first year, stomatal conductance (Cond(gs)) and quantum yield from CO_2 assimilation ($PhiCO_2$) were also affected by virus infection (Figure 2), while in the second-year transpiration rate (Trmmol) was significantly affected in plants infected with II and Q inocula (Figure 3). The negative effect of virus infection on grapevine photosynthesis corresponds to previous studies performed on other grapevine varieties [10,12,43]. Furthermore, in our study, plants infected only with GLRaV-3 (inoculum II) were not affected in the first year of the experiment by the reduction of phosphorus concentration and assimilation rate, unlike plants infected with other inocula, but only in the second year, proving the 'Tribidrag' response to virus infection depends not only on infection period but also on the virus composition of the inocula. Since the uptake of phosphorus can be limited in field conditions and mycorrhizal fungi are known to enhance the phosphorus uptake [44], it would be interesting to examine if mycorrhizal fungi could overcome such an effect of phosphorus deprivation in virus-infected plants and improve the photosynthesis performance.

Calcium deficiency was significant amongst all infected plants in the first year of measurement, which could be associated with the viral effect on phloem mobility since calcium mobility in *Vitis* is poor in general [41], and GLRaV-3 is known to cause particular damage in source-sink regulation in grapevine [45]. Due to the effects of GLRaV-3, sugars that are produced in leaves are not efficiently transported to the other parts of the plant [45]. This disruption in source-sink regulation in grapevine could also explain the deficiency of Mg in 'Tribidrag' plants infected with X, Y, and Q inocula. It is known that sugar over-accumulation in leaves is correlated to Mg deficiency [41]. Disruption in sugar transport from leaves is also proven by the gene expression analysis conducted in this study, where sucrose synthase 3 (SS3) was upregulated in the leaves of virus-infected plants (Figure 4). SS3 belongs to the family of SS enzymes that play a crucial role in a wide range of metabolic processes in plants such as starch biosynthesis, sucrose distribution between plant source and sink tissues, and response to biotic and abiotic stresses [46]. In the same study, Zhu et al. [46] proved that the SS3 gene, in particular, was upregulated in the case of various abiotic and phytoplasma-induced stresses, as well as GLRaV-3-induced stress on 'Cabernet Sauvignon' plants in the ripening stage.

Inoculum Y appears to have the most significant impact on 'Tribidrag' physiological processes, modifying the largest number of parameters measured at a significant level (10 in total) in the first year of the experiment. This inoculum is composed of two causal agents of grapevine leafroll disease, GLRaV-3 and GLRaV-1. 'Tribidrag' plants infected

with this inoculum were also the only ones developing typical GLD symptoms in both years of the experiment. Among other significant changes noted in this particular set of plants are significantly higher concentrations of Zn (also in GLRaV-3 singly infected plants) and Mn, along with significantly reduced concentrations of Cu. All of these elements play an essential role in reducing oxidative stress in a grapevine [41] that is known to be triggered by GLRaV-3 infection [15]. In the case of 'Tribidrag', it appears that copper, which is an important component of the superoxide dismutase enzyme [41], plays a lesser role in oxidative stress, being found in significantly lower concentrations in infected plants (Figure 2).

Symptoms on 'Tribidrag' plants were not very expressive (Supplementary Table S2), as only a few plants in two years of observations developed typical GLD symptoms, even though changes in grapevine physiology were noted. Similar findings were reported by Hančević et al. [15] for the same variety in a greenhouse, which is not uncommon in greenhouse conditions [47]. Furthermore, the 'Tribidrag' variety can also be frequently asymptomatic in field conditions even though some of its physiological processes are being significantly affected by GLD [48].

5. Conclusions

In this study, we confirmed the profound effect of grapevine viruses in the 'Tribidrag' variety, even though plants in most cases were asymptomatic. Phosphorus concentration along with photosynthesis-related parameters was affected by viral infection, indicating that the disturbance of photosynthesis is one of the most important plant mechanisms affected by virus infection. The response of 'Tribidrag' plants varies with the age of plants, as well as with the virus inocula used. Results obtained in this study confirm grapevine struggle in its interaction with viruses and the existence of host response even in the absence of visible symptoms. Virus-induced processes disturb the physiological balance of the grapevine and can significantly affect its performance, having important implications for the winemaking industry.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10050495/s1, Supplementary Table S1. Absorbance for ELISA test for GLRaV-3 on 'Tribidrag' plants used in the experiments. Absorbance is presented as mean values of two technical replicates., Supplementary Figure S1. Results of multiplex PCR obtained from samples of 'Tribidrag' plants infected with inoculum Z. For internal control, 18s genomic region of Vitis was used (844 bp). Products of grapevine leafroll-associated virus 3 (GLRaV-3) and grapevine virus A (GVA) are represented on the figure with their respective lengths: GLRaV-3–336 bp and GVA–272 bp., Supplementary Figure S2. Heat map analysis calculated from measured parameters in 'Tribidrag' plants infected with GLRaV-3 singly or in coinfection with other viruses (Table 1) in the second year of measurements, control plants are marked with C on the x axis. Distance was determined by Euclidean method (columns) and clustering (rows) was performed using Ward method [38]. Abbreviations are as following: c–concentration, Cu–Copper, Ca–calcium, Zn–zinc, Mn–manganese, P–phosphorus, Fe–iron, K_2O–Potassium, Mg–Magnesium, Car–Carotenoids, PhiCO_2–Quantum yield from CO_2 assimilation, DW leaves–dried weight of leaves, Photo (A)–assimilation rate, Trmmol–leaf transpiration, Cl a and b–Chlorophyll a and b, RWC–relative water content, Perm. Memb–Membrane permeability, Cond (gs)–stomatal conductance., Supplementary Figure S3. Results of ANOVA test and post hoc Tukey comparing the changes in measured parameters between control and infected plants in the first year of measurements. Significant changes are marked amongst boxplots representing individual treatments as listed in Table 1. Outlier measurements that are 1.5 times over the upper/lower quartile of the dataset of an individual boxplot are marked with points. Abbreviations for individual parameters are as following: c–concentration, P–phosphorus, K2O–potassium, Mg–magnesium, Ca–calcium, Zn–zinc, Mn–manganese, Cu–copper, Car–total carotenoid content, Photo (A)–assimilation rate, Cond (gs)–stomatal conductance, PhiCO_2–Quantum yield from CO_2 assimilation., Supplementary Figure S4. Results of ANOVA test and post hoc Tukey comparing changes in measured parameters in the infected with the control plants in the second year of measurements. Significant changes are marked amongst boxplots representing individual treatments

as listed in Table 1. Outlier measurements that are 1.5 times over the upper/lower quartile of the dataset of an individual boxplot are marked with points. Abbreviations for individual parameters are: c–concentration, P–phosphorus, Photo (A)–assimilation rate, Ci–Substomal CO_2 concentration, Trmmol–Leaf transpiration., Supplementary Table S2. List of abbreviations: physiological and morphological parameters as indicators of 'Tribidrag' response to viral infection., Supplementary Table S3. Raw measurements of physiological changes on 'Tribidrag' plants., Supplementary Table S4. Raw data on gene expression analysis on 'Tribidrag' plants.

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Data Availability Statement: Raw data obtained are available in Supplementary Table S3 for the physiological changes observed in 'Tribidrag' plants, while Ct values obtained in the gene expression study are available in Supplementary Table S4.

Conflicts of Interest: The authors declare no conflicts of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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