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Biochemical alternations in leaves of grapevine (*Vitis vinifera* L.) under virus-induced stress

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Abstract: Although GLD is widespread across major viticultural regions, its biochemical and physiological impacts on grapevine metabolism remain insufficiently characterized. This study aimed to detect GLRaV presence in grapevine cultivars from Azerbaijan and examine associated stress-related metabolic alterations. During the summer of 2023, a total of forty-eight symptomatic grapevine leaf samples were collected from vineyards in Salyan and Jalilabad regions. Visual symptoms included leaf rolling, interveinal reddening, mosaic patterns, and yield decline. Virus detection was performed using serological techniques (AgriStrip and DAS-ELISA) and molecular testing via RT-PCR. Results showed that 13 % of samples were infected with GLRaV-2 and 23 % with GLRaV-3. Biochemical profiling revealed increased malondialdehyde (MDA) accumulation, indicating elevated lipid peroxidation and oxidative stress in infected vines. Furthermore, significantly higher levels of total phenolic compounds, tocopherols, and soluble proteins were observed, suggesting enhanced non-enzymatic antioxidant responses. The activities of key antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX)—were markedly upregulated in virus-infected samples compared to healthy controls. These enzymatic changes highlight the grapevine's biochemical defense response to viral stress. Overall, the study demonstrates that GLRaV infection triggers both enzymatic and non-enzymatic antioxidant systems, which may serve as potential biomarkers for virus tolerance in grapevines.

Key words: ascorbate peroxidase (APX), catalase (CAT), Grapevine leafroll disease, lipid peroxidation, superoxide dismutase (SOD), tocopherols

Biokemične spremembe v listih žlahtne vinske trte (*Vitis vinifera* L.) pod stresom zaradi virusa

Izvleček: Čeprav je virus GLD zelo razširjen v večini vinogradniških regij, njegovi biokemični in fiziološki vplivi na metabolizem vinske trte ostajajo premalo opredeljeni. Namen te raziskave je bil odkriti prisotnost GLRaV v sortah vinske trte iz Azerbajdžana in preučiti s tem s stresom povezane presnovne spremembe. Poleti 2023 je bilo v vinogradih v regijah Salyan in Jalilabad zbranih skupaj oseminštirideset simptomatskih vzorcev listov vinske trte. Vizualni simptomi so vključevali zvijanje listov, pordelost medžil, mozaične vzorce in zmanjšanje pridelka. Detekcija virusa je bila izvedena s serološkimi tehnikami (AgriStrip in DAS-ELISA) in molekularnim testiranjem preko RT-PCR. Rezultati so pokazali, da je bilo 13 % vzorcev okuženih z GLRaV-2 in 23 % z GLRaV-3. Biokemijsko profiliranje je razkrilo povečano kopičenje malondialdehida (MDA), kar kaže na povečano peroksidacijo lipidov in oksidativni stres pri okuženih trtah. Poleg tega so opazili bistveno višje ravni skupnih fenolnih spojin, tokoferolov in topnih beljakovin, kar kaže na okrepljene neencimske antioksidativne odzive. Aktivnosti ključnih antioksidativnih encimov – superoksid dismutaze (SOD), katalaze (CAT) in askorbat peroksidaze (APX) – so bile v vzorcih, okuženih z virusom, opazno povečane v primerjavi z zdravimi kontrolami. Te encimske spremembe poudarjajo biokemični obrambni odziv vinske trte na virusni stres. Na splošno študija dokazuje, da okužba z GLRaV sproži encimske in neencimske antioksidativne sisteme, ki lahko služijo kot potencialni biomarkerji za toleranco vinske trte na virus.

Ključne besede: askorbat peroksidaza (APX), katalaza (CAT), bolezen zvijanja listov vinske trte, lipidna peroksidacija, superoksid dismutaza (SOD), tokoferoli

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1 INTRODUCTION

Grapevine (*Vitis* spp.) holds significant economic, cultural, and ecological value globally. As one of the oldest cultivated crops, it plays an essential role in the agricultural economies of many countries, contributing to the production of grapes for consumption, as well as for the wine industry, which is a multibillion-dollar global market (Gilardi et al., 2020). Grapes are not only appreciated for their nutritional benefits, such as being rich in antioxidants, vitamins, and minerals, but also for their commercial importance. According to the Food and Agriculture Organization (FAO), grapevines are grown in more than 60 countries worldwide, with the highest production levels seen in regions such as Europe, North America, and South America (Sultanova et al., 2019). In Azerbaijan, the cultivation of grapes has a long-standing tradition, and the country is known for producing a variety of indigenous grape cultivars, including those used for both table grapes and winemaking. The grape-growing regions of Azerbaijan, particularly in the foothills of the Caucasus and the Absheron Peninsula, benefit from the ideal climatic conditions necessary for high-quality grape production. The grapevine industry in Azerbaijan supports the local economy, creating jobs and promoting cultural heritage, while contributing to the global market of both table and wine grapes (Sultanova et al., 2024). Grapevine cultivation, like many other sectors of agriculture, is highly vulnerable to biotic stressors, among which viral diseases pose a particularly persistent and economically damaging threat (Maree et al., 2013). While fungal and bacterial pathogens contribute to disease pressure, viruses often go undetected for extended periods, silently impairing vine health and reducing productivity. These infections are primarily disseminated through insect vectors—such as mealybugs and aphids—as well as through vegetative propagation methods involving infected plant material (Montero et al., 2016). Among the various viral threats, Grapevine leafroll disease (GLD) stands out as one of the most destructive (El Aou-Ouad et al., 2016). Caused predominantly by Grapevine leafroll-associated viruses (GLRaVs), particularly GLRaV-2 and GLRaV-3, this disease is globally widespread and responsible for significant economic losses in viticulture (Fajardo et al., 2017). GLD manifests through leaf curling, interveinal reddening or chlorosis, delayed ripening, and decreased sugar accumulation in grape berries—ultimately compromising both yield and fruit quality (Luna et al., 2019). In addition to GLD, other important viruses such as *Grapevine fanleaf virus* (GFLV), *Grapevine virus A* (GVA), and *Grapevine virus B* (GVB) contribute to the complex viral disease landscape affecting vineyards worldwide. Although significant progress has been made, the etiology and symptom expression of GLD remain incompletely understood. This complexity arises because several virus species are associated with GLD, and symptom development is influenced by intricate interactions between biotic and abiotic factors. The disease tends to be more severe and apparent in cool-climate vineyards, where infected vines exhibit delayed fruit ripening, leading to reduced sugar accumulation and consequently diminished wine quality. The characteristic symptoms become most visible in autumn, particularly in red grape cultivars, which display interveinal reddening while the veins remain green. However, while many viral infections may initially remain asymptomatic or display only mild symptoms, their cumulative effects—especially under environmental stress—pose a serious threat to sustainable grape production.

Upon viral infection, plants experience a range of physiological disruptions, one of the most notable being oxidative stress (Topkaya, 2022; Topkaya et al., 2024). This stress arises due to an imbalance between the production of reactive oxygen species (ROS) and the plant's antioxidant defense capacity (Huseynova et al., 2014; Huseynova et al., 2018). ROS, such as superoxide radicals and hydrogen peroxide, are produced as byproducts of normal metabolic processes and environmental stress. Under normal conditions, plants have robust antioxidant defense systems to neutralize ROS, including enzymes like superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). However, viral infections, including those caused by GLRaV-2, exacerbate ROS production, leading to cellular damage, membrane lipid peroxidation, and DNA damage.

Antioxidant defense mechanisms play a critical role in mitigating oxidative damage during viral infection. Catalase (CAT) and ascorbate peroxidase (APX) are key enzymes involved in the breakdown of hydrogen peroxide, a harmful ROS (Bayramova et al., 2021). By catalyzing the conversion of hydrogen peroxide into water and oxygen, these enzymes help prevent oxidative damage to plant cells. Similarly, soluble proteins and phenolic compounds act as antioxidants, contributing to the overall defense mechanisms of the plant. The increase in antioxidant enzyme activity and the accumulation of phenolic compounds, such as flavonoids and tannins, are common responses to viral infections, reflecting the plant's adaptive strategy to cope with oxidative stress. Viral infection in grapevines has a profound effect on various metabolic processes, particularly those related to oxidative stress and secondary metabolism (Bertamini et al., 2004). The infection by GLRaV-2 leads to changes in the levels of tocopherols,

proteins, and phenolic compounds. Tocopherols, also known as vitamin E, are important antioxidants that protect cell membranes from oxidative damage. The increase in tocopherol levels observed in GLRaV-2-infected grapevines reflects the plant's attempt to mitigate oxidative stress. Similarly, the increase in soluble proteins in infected plants suggests an upregulation of defense-related proteins in response to the pathogen (Christov et al., 2006). These proteins may include chaperones, heat shock proteins, and enzymes involved in secondary metabolism. Moreover, the accumulation of phenolic molecules possessing free radical-scavenging properties, is a well-documented response to pathogen infection in plants. The elevated levels of phenols in GLRaV-2-infected grapes indicate an enhanced defense response to the virus. Additionally, viral infection leads to lipid peroxidation, a process where ROS degrade cellular lipids, causing membrane damage and affecting cell integrity. This process is often measured by the increase in malondialdehyde (MDA), a byproduct of lipid peroxidation, which serves as an indicator of oxidative damage in plant cells. This study aims to explore the effects of virus infection on grapevine metabolism, with a particular focus on the oxidative stress responses.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

The main part of the research was carried out in the Bioadaptation Laboratory at the Institute of Molecular Biology and Biotechnologies, Ministry of Science and Education of the Republic of Azerbaijan. Surveys were carried out in 2023 to identify GLD symptoms in the major grapevine-growing regions of Azerbaijan, specifically from Salyan and Jalilabad. During the monitoring, a total of 48 plant samples were identified by visual diagnosis according to characteristic symptoms of closteroviruses. The collected leaf samples were carefully transported to the laboratory and stored at 4 °C until further processing. Healthy and completely virus-free plant leaves were selected as the reference group.

2.2 GLRAV-3 AND GLRAV-2 IDENTIFICATION

According to the manufacturer's instructions collected samples were analyzed for the presence of GLRaV-3 virus using rapid one-step assay AgriStrip, DAS-ELISA (double antibody sandwich enzyme-linked immunosorbent assay), and RT-PCR (Reverse transcription-polymerase chain reaction). Initially, plant extracts were subjected to incubation with magnetic beads that were coated with antibodies specific to the pathogen (Bioreba, Reinach, Switzerland). Subsequently, these beads were isolated from the extract using a magnet and reconstituted in a minimal volume of running buffer. Following this, a strip was introduced, and the concentrated beads traversed upward through the strip, resulting in the appearance of brown-colored lines. Positive extracts, containing the pathogen, exhibited visibility of both test and control lines, while negative samples displayed solely the upper control line. The coloration reached a high intensity within 10-20 minutes, and the outcomes were recorded. The initial assessment for the presence of GLRaV-3 and GLRaV-2 in the gathered grapevine samples also was performed by DAS-ELISA, following the methodology outlined in reference (Bayramova et al., 2021) and the examination was carried out according to the guidelines provided by the antisera producer (Bioreba, Reinach, Switzerland). The enzymatic reaction occurring on the ELISA plate was first assessed by color change. Absorbance at 405 nm was measured more than two times with an ELISA reader, Stat Fax Microplate, Awareness Technology, USA.

2.3 ENZYME ACTIVITY MEASUREMENT

2.3.1 Preparation of enzyme extract

For enzymatic analysis, 0.5 g of grapevine leaf tissue was rapidly ground in liquid nitrogen and suspended in an extraction buffer consisting of 100 mM sodium phosphate (pH 7.8), 1 mM EDTA, 2 mM PMSF (phenylmethylsulfonyl fluoride), 1 % PVP (polyvinylpyrrolidone), and 0.1 % Triton X-100. The homogenate was centrifuged at 15,000 × g for 20 minutes at 4 °C. The supernatant was collected and used immediately for enzymatic activity assays of SOD, CAT, and APX.

2.3.2 Ascorbate peroxidase (APX, EC 1.11.1.11)

APX activity was quantified by monitoring the decline in absorbance at 290 nm, following the oxidation of ascorbate. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.6), 0.1 mM EDTA, 0.05 mM ascorbate, 0.1 mM hydrogen peroxide (H_2O_2), and 100 µl of enzyme extract in a total volume of 1 ml. Measurements were recorded using a UV–visible spectrophotometer (Ultrospec 3300 Pro, Amersham Biosciences, USA). The molar extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate enzyme activity, which was ex-

pressed as µmol ascorbate oxidized per mg protein per minute [µmol mg^{-1}(protein) min^{-1}].

2.3.3 Catalase (CAT, EC 1.11.1.6)

CAT activity was measured by assessing the decomposition of hydrogen peroxide (H_2O_2) at 240 nm. For extraction, 1 g of leaf material was homogenized in 10 ml of 50 mM potassium phosphate buffer (pH 7.0), filtered, and centrifuged at 8000 × g for 10 minutes. The reaction mixture contained 2.9 ml of phosphate buffer, 25 µl of enzyme extract, and 90 µl of 3 % H_2O_2 to initiate the reaction. The reduction in absorbance was recorded spectrophotometrically. Enzyme activity was determined using the extinction coefficient 39.4 mM^{-1} cm^{-1} and expressed as [µmol mg^{-1}(protein) min^{-1}].

2.3.4 Superoxide Dismutase (SOD, EC 1.15.1.1)

SOD activity was evaluated using the SOD Assay Kit-WST (Sigma-Aldrich, USA), which quantifies superoxide radical scavenging. Fresh leaves were homogenized in 50 mM potassium phosphate buffer (pH 7.8), and the resulting extract was centrifuged to obtain the cytosolic enzyme fraction. The inhibition of formazan dye formation was monitored at 450 nm using a microplate reader. The activity was calculated based on the inhibition rate and reported as [µmol mg^{-1}(protein) min^{-1}].

2.4 TOCOPHEROL DETERMINATION

Tocopherol was estimated in the plant samples by the Emmerie-Engel reaction. It is based on the reduction of ferric to ferrous ions by tocopherols, which, with 2, 2'-dipyridyl, forms a red color. Tocopherols and carotenes are first extracted with xylene and read at 460 nm to measure carotenes. A correction is made for this after adding ferric chloride and reading at 520 nm. Tocopherol was used as a standard. The concentration of tocopherol in the sample was calculated using this formula:

Tocopherols (µgmg^{-1} fresh mass) = (Sample A520-A460)/Standard A520×0.29× 0.15

2.5 DETERMINATION OF LIPID PEROXIDATION

The intensity of the lipid peroxidation process in plants was calculated in terms of MDA in healthy and infected leaf samples. The amount of MDA is determined by a spectrophotometric method based on the reaction of thiobarbituric acid at 532 and 600 nm.

2.6 DETERMINATION OF TOTAL SOLUBLE PROTEIN (TSP) CONTENT

The total soluble protein (TSP) content in grapevine leaf extracts was determined using the Bradford method, a rapid and sensitive spectrophotometric assay based on protein-dye binding, originally described by Bradford (1976). The reaction mixture was prepared by combining 100 µl of the leaf extract supernatant with 2.0 ml of 0.12 % Coomassie Brilliant Blue G-250 reagent. After thorough mixing, the absorbance of the resulting solution was measured at 595 nm using a UV-Vis spectrophotometer. To ensure accurate quantification, a standard calibration curve was constructed using bovine serum albumin (BSA) in the concentration range of 1200–1600 µg g^{-1} fresh mass as the reference protein. All samples were analyzed in triplicate, and TSP content was expressed in µg per gram of fresh leaf tissue.

2.7 TOTAL PHENOLIC CONTENT

The total phenolic content in both infected and non-infected grapevine tissues was quantified following the method described in Ref. (Sultanova et al., 2019). This assay is based on the reaction of phenolic compounds with phosphomolybdic acid in the Folin-Ciocalteu reagent, resulting in the formation of a blue-colored complex in an alkaline medium. The absorbance of this complex was measured spectrophotometrically at 650 nm. A calibration curve was generated using a gallic acid standard solution (0.2–1.0 ml), corresponding to concentrations ranging from 2.0 to 10 µg. The total phenolic content was expressed as mg g^{-1} of dry mass.

2.8 STATISTICAL ANALYZES

The collected experimental data for all evaluated physiological and biochemical parameters were subjected to rigorous statistical analysis. Analysis of Variance (ANOVA) was performed using CoStat software (version 6.303, USA) to determine the significance of treatment effects at $p \leq 0.05$. Mean comparisons were conducted using the Least Significant Difference (LSD) test, allowing differentiation of treatments with statistically significant variations. To further explore relationships among measured variables and treatments, multivariate statistical techniques were applied using R Studio (version 4.2.2). All graphical outputs, including cluster dendrograms, PCA biplots, and heatmaps, were created in R Studio for an enhanced interpretation of the interrelationships

among measured parameters under virus-induced stress conditions.

3 RESULTS AND DISCUSSION

During the summer of 2023, a total of forty-eight grapevine samples exhibiting characteristic virus-related symptoms—such as leaf curling, vein greening, reduced leaf size, necrosis, mosaic patterns, reddening, and a decline in fruit yield and quality—were collected from various regions of Azerbaijan (Figure 1). To determine the presence of major grapevine-infecting viruses, these samples underwent both serological analysis (using the rapid one-step Agri Strip assay and DAS-ELISA) and molecular detection via RT-PCR.

The findings indicated that 13 % of the analyzed samples (6 out of 48) tested positive for GLRaV-2. Furthermore, GLRaV-3 was identified in 11 samples, accounting for an infection rate of 23 %.

The reliability of the DAS-ELISA findings was further corroborated through RT-PCR analysis, using specific primers targeting partial sequences of the coat protein (CP) genes. Notably, grapevine plants that exhibited no visible symptoms did not test positive for GLRaV-3 or GLRaV-2 through either serological or molecular methods. Conversely, all samples that were identified as virus-positive via DAS-ELISA were also confirmed through RT-PCR, demonstrating the consistency and accuracy of both diagnostic approaches. No viral RNA amplification was observed in RT-PCR assays conducted on leaf extracts from virus-free grapevines, further validating the specificity of the detection methods. Although RT-PCR was employed to confirm the presence of viral infections, the corresponding gel electrophoresis data were not included in the manuscript.

Understanding plant-pathogen interactions and the molecular mechanisms underlying plant responses to viral infections requires an in-depth examination of virus-induced metabolic alterations. Investigating these biochemical changes provides critical insights into the adaptive strategies of grapevines in response to infection and their potential implications for disease resistance and management strategies. Biochemical analyses were subsequently performed on both virus-infected grapevine plants and non-infected (healthy) controls.

Malondialdehyde (MDA) is a widely recognized biomarker for assessing lipid peroxidation in plant tissues, serving as an indicator of oxidative stress intensity. Elevated MDA levels are commonly associated with increased cellular damage resulting from stress conditions. In this study, a substantial rise in MDA content—approximately 2.0 to 2.2-fold was observed in the leaves of virus-infected grapevines compared to the control plants, suggesting heightened oxidative stress and potential membrane damage. Similar results were noted in leaves inoculated with *Bean yellow mosaic virus*, as reported by Karar A. Hamzah in 2021. This research aimed to evaluate lipid peroxidation and total phenolic content (TPC) in fava bean plants affected by *Bean yellow mosaic virus* and investigate the effectiveness of biostimulants in managing the infection. The results indicated that virus-infected plants experienced heightened stress, as evidenced by increased malondialdehyde (MDA) levels, whereas other treatments led to a reduction in MDA content. Notably, their observations indicated a progressive rise in MDA accumulation levels over time in the inoculated plants, with markedly higher levels compared to the control group. Previous studies explored the mechanism

Figure 1: Characteristic symptoms of Grapevine Leafroll Disease (GLD) in infected grapevine leaves.

wherein free radicals and H_2O_2 acquire electrons from lipid molecules within the cell membrane, ultimately leading to lipid peroxidation. Consistent with these findings, our results (Figure 2) revealed a marked increase in malondialdehyde (MDA) levels in all virus-infected grapevine samples, reaching 2.2 and 2.5 mmol·mg^{-1} FM, respectively, compared to 1.2 mmol·mg^{-1} FM in the healthy control group.

In this study, the activities of key antioxidant enzymes—superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX)—significantly increased in grapevine leaves infected with GLRaV-2 and GLRaV-3 compared to healthy controls. The control samples exhibited basal enzyme activities (SOD: 0.82, CAT: 0.65, APX: 0.74 µmol mg^{-1}(protein) min^{-1}), whereas virus-infected samples showed a notable upregulation, reaching up to 2.35 µmol mg^{-1}(protein) min^{-1} for SOD, 2.40 for CAT, and 2.30 for APX (Figure 2). This upsurge in enzymatic activity suggests an enhanced oxidative burst triggered by virus-induced stress. The increase in SOD activity reflects the plant's need to neutralize elevated superoxide radicals O_2^- a common consequence of pathogen interaction. SOD converts these radicals into H_2O_2, which is subsequently broken down by CAT and APX, indicating a coordinated antioxidant defense strategy. The significant rise in CAT and APX levels supports the hypothesis that grapevine tissues activate both H_2O_2-detoxifying systems to mitigate oxidative damage and maintain redox balance under virus stress. Similar results have been reported in other plant-virus interactions. For example, Islam et al. (2020) observed elevated SOD and CAT activities in *Tomato yellow leaf curl virus* (TYLCV)-infected plants, indicating oxidative stress-induced activation of antioxidant enzymes. Similarly, Ramzan et al. (2021) reported that bacterial spot disease in *Capsicum annuum* L. cultivars triggered increased antioxidant enzyme and isoform activity as part of the plant's stress response. Likewise, Huang et al. (2018) demonstrated a significant upregulation of APX activity in rice infected with *Rice stripe virus* (RSV), suggesting enhanced tolerance mechanisms. The enzymatic responses observed in the present study are in agreement with those reported by Vitti et al. (2013), who documented increased ROS-scavenging enzyme levels in citrus plants infected with *Citrus tristeza virus* (CTV). These findings are further supported by Miras-Moreno et al. (2022) and Sharma et al. (2024), who noted similar antioxidant defense responses in grapevine under abiotic stress conditions.

These findings reinforce the role of antioxidant enzymes as key markers in plant stress physiology and highlight their potential utility in breeding programs aimed at enhancing virus tolerance in grapevines.

The non-enzymatic antioxidants play a crucial role in maintaining the balance of redox processes in plants. It is well-established that the transmission of signals through reactive oxygen species (ROS) plays a crucial role in the plant's resistance to pathogens. Upon infection, the activity of antioxidant enzymes acts to block damaged zones in plants, leading to an increase in the levels of phenolic compounds. Consequently, plants have evolved well-coordinated antioxidant defense systems to manage oxidative stress. Tocopherols, owing to their antioxidant activity, play a crucial role in conferring tolerance to various biotic and abiotic stresses such as salinity, drought, metal toxicity, ozone, and UV radiation. Tocopherols exhibit diverse functions in plant growth and

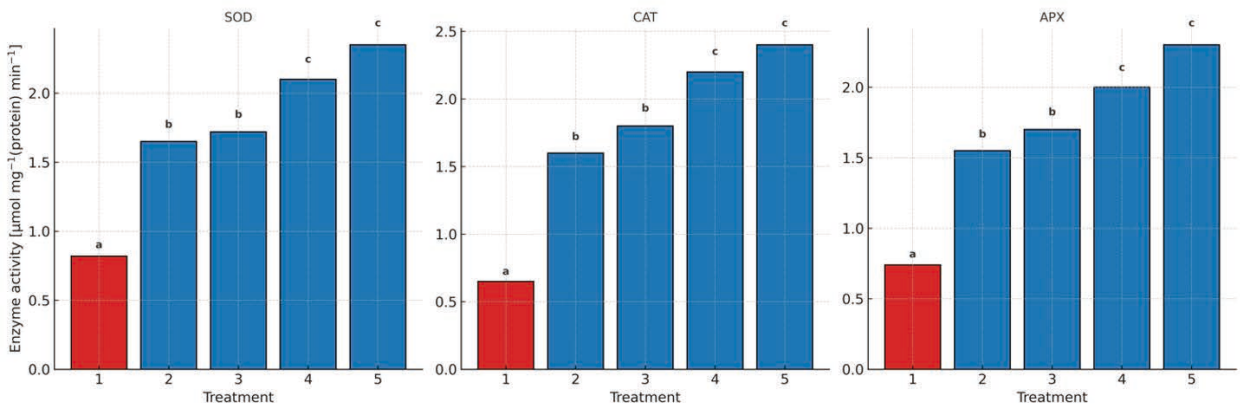

Figure 2: Activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) in healthy and virus-infected grapevine leaves. Treatment groups are as follows: (1) Healthy control (red), (2–3) GLRaV-2 infected, and (4–5) GLRaV-3 infected (blue). Bars represent mean values with standard deviations. Different letters above the bars indicate statistically significant differences among treatments based on analysis of variance (ANOVA), followed by the LSD test at $p \leq 0.05$ (CoStat v6.303, USA). Enzyme activity is expressed in [µmol mg^{-1}(protein) min^{-1}].

physiological processes, impacting overall yield. They belong to a distinct group of potent antioxidants and play a pivotal role in signaling and gene expression. Our studies revealed a noteworthy increase in the tocopherol content in all infected grapevine leaves (Figure 3). Specifically, the levels of tocopherols (vitamin E) in the infected leaves ranged from 1.24 to 1.54 μg mg^{-1} fresh mass, respectively.

As is well established, phenolic compounds representing a major class of metabolites contributing to antioxidant defense mechanisms under various stress conditions. In this study, the total phenolic content (TPC) was significantly elevated in the leaves of both virus-infected investigated grapevine samples. Specifically, TPC levels ranged from 88.78 to 92.45 mg g^{-1} dry mass in infected GLRaV-2 leaves and from 86.23 to 79.96 mg g^{-1} dry mass in infected GLRaV-3 leaves (Figure 2). Notably, the TPC content in infected GLRaV-2 samples was 1.6 times higher compared to the healthy controls, while infected GLRaV-3 samples exhibited a 1.5-fold increase in phenolic accumulation, indicating a substantial metabolic response to viral infection.

Abiotic and biotic stress factors, which are among the major environmental challenges affecting plant metabolism, are known to collectively influence the accumulation of soluble sugars and proteins (Desbiez et al., 2019). The total soluble protein content, as presented in Figure 3, demonstrates a significant increase in response to virus in grapevine leaves both GLRaV-2 and GLRaV-3 infection.

Specifically, infected GLRaV-3 leaves exhibited a 19.42 % increase in total soluble protein content compared to healthy controls. These findings suggest that viral infection induces metabolic adjustments in grapevines, likely as part of a defense mechanism against pathogen-induced stress.

In order to better understand the interrelationships among physiological and biochemical parameters under virus-induced stress, multivariate statistical analyses were conducted. The heatmap correlation matrix revealed strong positive associations among most traits,

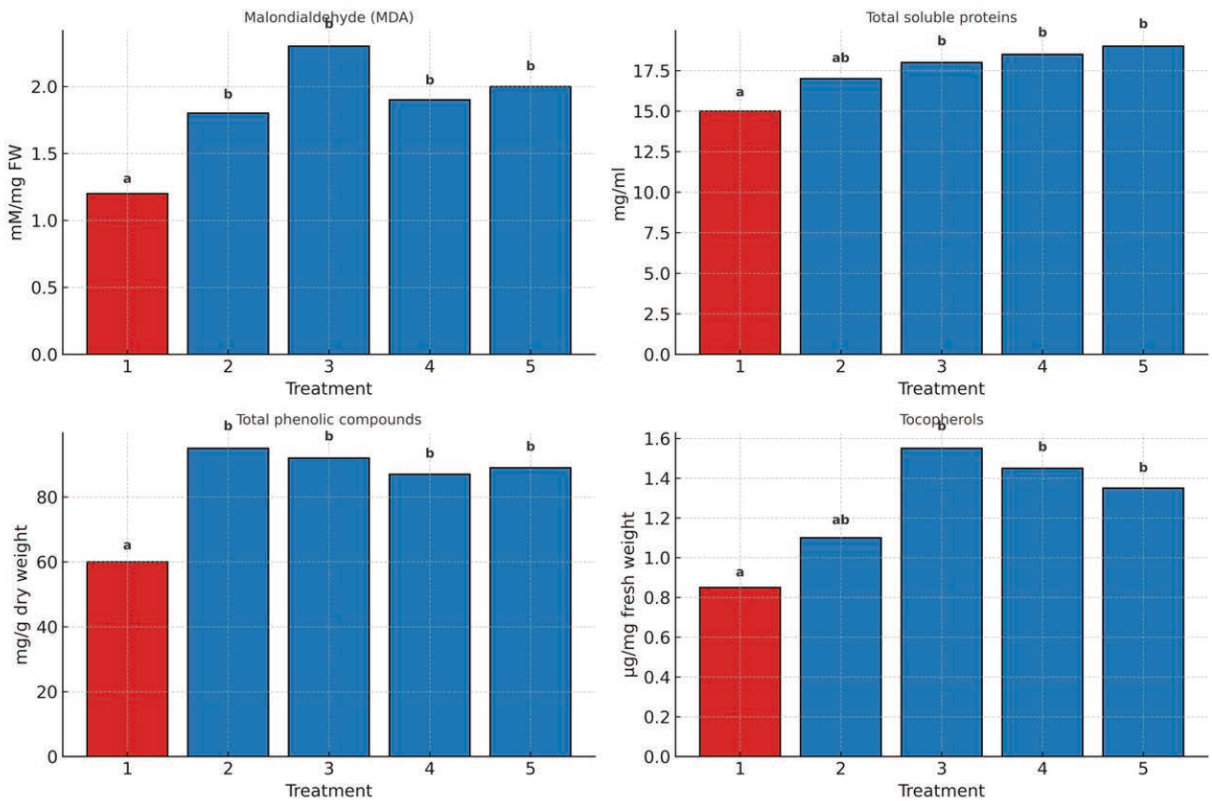

Figure 3: Comparative analysis of malondialdehyde, total phenolic compounds, total soluble proteins, and tocopherol content in healthy and virus-infected grapevine leaves. Samples were categorized as follows: (1) Healthy control, (2–3) Grapevine leaves infected with GLRaV-2, and (4–5) Grapevine leaves infected with GLRaV-3. Bars represent mean values with standard deviations. Different letters above the bars indicate statistically significant differences among treatments according to ANOVA followed by LSD test at $p \leq 0.05$ (CoStat v6.303, USA).

indicating a tightly regulated defense response triggered by viral infection (Figure 4).

Specifically, malondialdehyde (MDA)—a key marker of lipid peroxidation—showed strong positive correlations with total phenolic compounds (r = 0.91), tocopherol content (r = 0.85), and soluble proteins (r = 0.80), suggesting that oxidative stress leads to the accumulation of protective secondary metabolites and stress-related proteins. Importantly, antioxidant enzyme activities also demonstrated meaningful associations with biochemical parameters. For instance, superoxide dismutase (SOD) activity was highly correlated with MDA (r = 0.89), indicating that superoxide radical formation due to lipid peroxidation strongly stimulates SOD activity. Catalase (CAT) and ascorbate peroxidase (APX) also exhibited positive relationships with both MDA and phenolic content, supporting their role in scavenging hydrogen peroxide and reinforcing redox homeostasis.

These coordinated changes suggest that viral infection elicits a systemic oxidative response that activates both enzymatic and non-enzymatic antioxidant defense systems. The cluster dendrogram further supports these findings, with antioxidant enzymes (SOD, CAT, APX) clustering closely with MDA and phenolics, highlighting their functional interdependence in mitigating oxidative stress. Such multilevel coordination among physiological, biochemical, and enzymatic responses underscores the complexity of grapevine defense mechanisms and may provide insight into biomarkers for stress tolerance or targets for breeding virus-resistant cultivars.

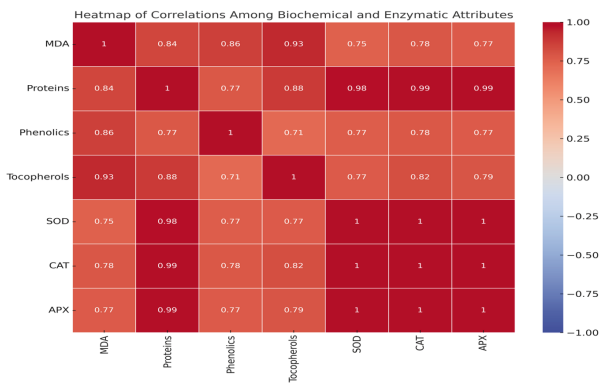

Figure 4: Heatmap visualization showing Pearson correlation coefficients among physiological and biochemical attributes of grapevine leaves under virus-induced stress. Strong positive correlations were observed between malondialdehyde (MDA), phenolic compounds, tocopherol content, and soluble proteins, indicating coordinated stress response mechanisms. Color intensity represents the strength and direction of the correlation (red: positive; blue: negative). Asterisks denote statistically significant correlations ($p \leq 0.05$).

The cluster dendrogram analysis further supported these findings by grouping the variables into closely associated clusters (Figure 5). Tocopherols and total soluble proteins formed one cluster, reflecting their functional relationship in cellular protection and stress tolerance. MDA and total phenolic compounds clustered separately but remained in close proximity, suggesting that phenolic compounds may serve as a direct antioxidant response to virus-induced lipid peroxidation. Interestingly, antioxidant enzymes—SOD, CAT, and APX—also formed a distinct yet related cluster, positioned near the MDA and phenolic group. This spatial association highlights the role of enzymatic antioxidants in mitigating reactive oxygen species (ROS) generated during oxidative stress.

The proximity of SOD to MDA reinforces its primary function in neutralizing superoxide radicals formed during membrane lipid damage. Meanwhile, CAT and APX, responsible for H_2O_2 detoxification, clustered nearer to phenolics and proteins, implying a coordinated function with non-enzymatic antioxidants in maintaining redox balance. This clustering pattern illustrates that enzymatic and non-enzymatic antioxidant systems operate in an interconnected network, responding synergistically to viral stress. Such integration of physiological, biochemical, and enzymatic responses reveals the complex defensive architecture activated in grapevines to minimize virus-induced cellular damage.

Together, these multivariate analyses highlight the integrated nature of grapevine biochemical responses to GLRaV infection. The observed associations support the hypothesis that oxidative stress markers, antioxidant capacity, and protein metabolism are tightly regulated in response to viral pressure. These findings contribute to a more holistic understanding of plant-virus interactions

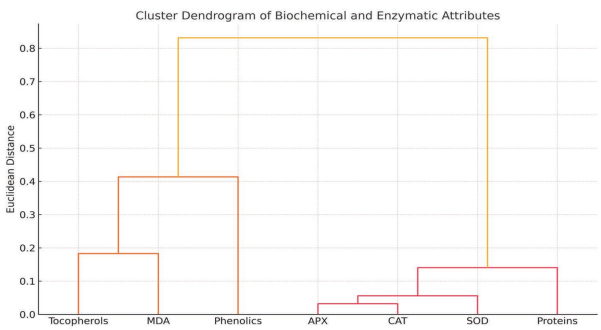

Figure 5: Hierarchical cluster dendrogram of physiological and biochemical parameters in virus-infected grapevine leaves. Clustering was performed using Ward's method and Euclidean distance. The dendrogram groups closely associated traits, highlighting functional relationships among oxidative stress markers, antioxidants, and protein content in response to GLRaV infection.

and could be useful in identifying key biomarkers for early detection and breeding of virus-tolerant grapevine cultivars.

Both GLRaV-2 and GLRaV-3, members of the Closteroviridae family, were detected in symptomatic grapevine samples and are known to induce substantial physiological and metabolic disruptions in infected plants. These phloem-limited viruses, characterized by their long, flexuous, positive-sense single-stranded RNA genomes (~18.5–19.3 kb), interfere with normal phloem function, thereby impairing nutrient translocation and altering host metabolism. In this study, GLRaV-2 and GLRaV-3 infections were associated with classic leafroll symptoms—such as leaf curling, interveinal reddening, and delayed ripening—particularly in red-leafed cultivars. The observed decline in photosynthetic efficiency and fruit quality in infected vines is consistent with previous reports highlighting GLRaV-mediated reductions in chlorophyll content and increased oxidative stress (Fajardo et al., 2017; Luna et al., 2019). These effects are partly attributable to the virus-induced modulation of host cellular machinery during replication and systemic movement. Moreover, the viral replication process within the phloem is linked to elevated oxidative damage, as evidenced by increased malondialdehyde (MDA) levels and upregulation of both enzymatic (SOD, CAT, APX) and non-enzymatic antioxidants (phenolics, tocopherols).

The genetic diversity of GLRaV-3, in particular, likely contributes to variable symptom expression and differential biochemical responses observed among infected plants. Its widespread dissemination via infected propagation material and vector transmission complicates control efforts and underscores the need for virus indexing and the development of resistant cultivars. Overall, the molecular and physiological evidence presented here highlights the critical impact of GLRaVs on grapevine health and metabolism.

4 CONCLUSION

Grapevine leafroll-associated viruses GLRaV-2 and GLRaV-3 pose serious threats to viticulture by significantly disrupting vine physiology, fruit quality, and key metabolic processes. Their confinement to the phloem, combined with efficient spread via insect vectors and infected propagation materials, complicates control efforts. Infected vines exhibit heightened oxidative stress, which triggers complex antioxidant defense responses aimed at minimizing damage. This study underscores the substantial biochemical alterations induced by these viruses and sheds light on the plant's adaptive metabolic responses. A clearer understanding of these physiological and biochemical shifts can guide the development of integrated management strategies—such as the use of virus-resistant cultivars, targeted vector control, and antioxidant-based treatments. Moving forward, deeper investigation into the molecular mechanisms driving these metabolic changes will be essential for enhancing disease resistance and resilience in grapevine breeding programs.

Research data availability statement

All research data are included in manuscript.

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