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Research Paper

# Changes in the Concentrations of Primary and Secondary Metabolites Involved in the Infection Process by *Grapevine leafroll associated virus* and *Grapevine virus A* in *Vitis vinifera* L.

**Sofía Murúa[1*], Daniel Bustos[2]**

[1,2]Instituo de Ciencias Básicas, Universidad Nacional de San Juan, San Juan, Argentina

**Corresponding Author: sofimurua@gmail.com*

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***Abstract***— Grapevine Leafroll desease (GLD) is among the most important diseases that affect the vine, which cause significant economic losses in cultivars of these plant species, since they cause low-quality berries, in terms of their size and grade. sugar. The changes suffered by plants at the metabolic level were studied in the present work. Four plants of *V. vinifera* L. cv. Thompson Seedless, one infected only with GLRaV-3, another infected with GLRaV-3 coinfected with and GLRaV-2, a third plant infected with GLRaV-2 coinfected with GLRaV-3 and GVA, and finally a plant that did not show viral titer (used as negative control), all from the 2018-2019 harvest, from a commercial cultivar located in the province of San Juan, Argentina. Changes in α-tocopherol contents, antioxidant capacity, total polyphenols, soluble sugars, soluble proteins, proline and glycine betaine were analyzed, in addition to studying the increase in resveratrol as induced phytoalexin. This can be considered as a plant adaptation mechanism to live with a viral pathogen.

***Keywords***— Phytopathology, *Vitis vinífera*, Secondary Metabolism, Grapevine viruses (GLRaV)

## 1. Introduction

*Vitis vinifera* L. is affected by various pathogenic microorganisms that cause damage to foliage, roots, and wood. Among the causative agents, fungi, bacteria, viruses and nematodes can be mentioned, which can cause significant losses in yield, as well as in the commercial quality of table grapes [38], [70]. These pathogenic agents can affect plants in different phenological stages and reduce their useful life, as well as reduce the postharvest quality of their fruits [13]. Depending on the incidence and severity of the phytopathological problems, these can become limiting factors for production, causing economic losses to producers and difficulties in marketing and export [47], [49]. The incidence and severity of each disease will vary according to the climatic characteristics that occur in each region. In localities that present conditions of high humidity or free water during the growing season, diseases can be the main limitation of the crop in economic terms [53], [55].

Among the viral diseases are the Grapevine Leafroll Disease (GLD), caused mainly by the *Grapevine leafroll-associated virus*-3 (GLRaV-3), this disease is mainly characterized by having a negative impact on the quality of the fruit, reducing its size and sugar grade, considerably decreasing its quality [2].

Vine roll is the most widespread viral disease in the world that affects the vine, causing great economic losses, as a consequence of the drop in yield, which is due to the drop in the weight of the berries and the sugar content of the fruit [48], [54].This disease is estimated to cause a loss of between 25,000 and 40,000 dollars per hectare of 25-year-old vineyards [3]. The symptoms vary according to the type of cultivar and the different viral combinations that affect them [50], [51] This disease can be caused by a simple or mixed virus infection [48], the predominant virus being *Grapevine leafroll-associated virus* -3 (GLRaV-3) [50], the viruses with which it is associated in mixed infection can be *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll associated virus*-1 and 2 (GLRaV-1 and GRAV-2), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine rupestris stem pitting associated virus* (GRPSaV) and *Grapevine fleck virus* (GFkV) [24], [40].

The biochemical differentiation that exists between an infected and a healthy vine plant occurs at different levels of their metabolic processes [4]. The biosynthetic pathway of phenylpropanoids in grapevine is one of the most studied and related to plant defense mechanisms against a wide range of stress factors (biotic and abiotic), and is responsible for the synthesis of a large number of phenolic compounds [19],[35].

 

## 2. Related Work

A large amount of previous research has demonstrated the negative effect of viral infections on the metabolism of the host plant. For example, in an investigation carried out on potato plants (*Solanum tuberosum* L.) infected with *Potato leafroll virus* and *Potato virus Y*, it was observed that the sprouts increased the levels of chlorophyll, soluble proteins and total sugars as consequence of the physiological changes caused by the viral load in the different organs of the plant [43], [17].

In the same way, changes at the metabolic level have been reported in plants infected with ZYMV (*Zuccini yellow mosaic virus*) [61].

Another clear example is the research on the effects on the expression in the secondary metabolisms of *Nicotiana bentamiana* when it is attacked by *Potato virus* X, where it is shown that it expresses genes belonging to this secondary metabolism, resulting in the synthesis and accumulation of a terpenoid phytoalexin [44].

## 3. Experimental Method

### 2.1) CHEMICAL REAGENTS AND SOLVENTS

The pure grade solvents were distilled before their use, those of analytical grade and High Performance Liquid Chromatography (HPLC) grade were used as they were provided by the supplier SALPER S.A, Mendoza headquarters. The standard reagents and compounds used are mentioned below: β-carotene (Supelco, China), α-tocopherol (Supelco, USA), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma, China), gallic acid (Sigma, USA), citric acid (Supelco, USA), D-(-)-fructose (Sigma aldrich, USA), Coomassie brilliant G-250 (Biopack, Argentina), Bovine serum albumin (Roth, Germany), Ninhydrin (Merck, USA) , sulfosalicylic acid (Biopack, Argentina), L-proline (Biopack, Argentina), glycine betaine (Sigma, USA), 1,2-dichloroethane (PHYW, Germany), o-phenanthroline (Anhedra, USA), Folin-Reagent Ciocalteu (Biopack, Germany), anhydrous sodium carbonate (Biopack, Argentina), Phenol (Biopack, Argentina), phosphoric acid 85% (Tetrahedron, Argentina), potassium iodide (Anhedra, Argentina), resublimated iodine (BIOPACK, Argentina) . TROLOX (Calbiochem, Germany).

### 2.2) PLANT MATERIAL AND VIRAL IDENTIFICATION.

Leaf samples were taken from four *Vitis vinifera* L. cv Thompson Seedless plants during the prevalence of the viral infection, in the month of January 2019. The commercial cultivation of the variety is located in the Albardón department, in the province of San Juan, Argentina. During harvest, leaves were selected from plants that showed characteristic symptoms of leaf roll disease, such as leaf chlorosis, inward rolling, and low-quality berries. Leaves, nodes, tendrils and petioles were collected and transported to the laboratory on ice at a temperature of 5 $^0$C and stored until processing. Viral detection in the selected plants was carried out at the National Institute of Agricultural Technology, Experimental Station located in the province of Mendoza. They were carried out by means of RT-PCR analysis containing specific primers for the GVA, GLRaV-1, 2 and 3, and GFkV viruses.

### 2.3) EXTRACTION AND QUANTIFICATION OF METABOLITES INVOLVED IN THE INFECTIOUS PROCESS

#### 2.3.1) EXTRACTION AND ESTIMATION OF A-TOCOPHEROL

The extraction and determination process was carried out using the method described by Martinek, 1968. 5 g of fresh leaf were chopped, and proceeded to extract with 30 ml of xylene in a sonicator for 15 minutes at room temperature, then filtered, in order to separate the leaves from the solution, through Whatman N$^o$ 40 filter paper, permanently protecting from light and proceeding to the determination immediately to avoid oxidation of the extracted analyte. We proceeded to work with 1 ml of filtrate, whose absorbance is read at 460 nm (Abs sample 460 nm). Then, 1 ml of 0.12% $FeCl_3$ solution in absolute ethanol and 0.5 ml of 0.1% O-phenanthroline in absolute ethanol were added, vortexed for 30 seconds, then centrifuged for 5 minutes at 350 rpm and finally the absorbance at 600 nm of the upper layer was read (Abs sample at 600 nm). A solution of standard α-tocopherol in xylene at a concentration of 0.5 mg/ml is used for the procedure, treating it in the same manner mentioned above, in order to read the absorbance at 600 nm (Abs. Standard Sol 600 nm). The concentration of α-tocopherol in each sample is calculated using the following formula:

$$\text{Conc. of } \alpha\text{-tocoferol } (\mu g/mg\ PF) = \frac{\text{Abs sample 600 nm} - (0{,}373 \times \text{Abs sample 460 nm})}{\text{Abs. standar 600 nm}}$$

#### 2.3.2) ESTIMATION OF ANTIOXIDANT COMPOUNDS

The total antioxidant activity of the methanolic fraction obtained was studied using the DPPH method proposed by Bakhta et al (2016) and Prior et al (2005). Briefly, 250 µl aliquots of each extract were brought to 0.5 ml with methanol and then 1 ml of the 6 x $10^{-6}$ M concentration DPPH solution. After 20 minutes, at a temperature of 25 ºC, the decrease in absorbance at 517 nm was determined, in the dark. As a positive control, 1% ascorbic acid in methanol was used. The percentage inhibition of the DPPH radical was calculated using the following formula:

$$\%\ \text{inhibición} = (A_0 - A_1 / A_0) \times 100$$

Using a calibration curve made with TROLOX as a base, the result of the antioxidant capacity is expressed as micrograms of TROLOX per milliliter of extract from each plant, thus expressing the amount of antioxidant necessary to reduce the initial concentration of antioxidants by 50%. DPPH ($EC_{50}$).

 

2.3.3) ESTIMATION OF SOLUBLE SUGARS

To extract the soluble sugars, the method proposed by Martin et al, 2000, with some modifications, was followed. 1 g of dry and chopped leaf was macerated with a mortar in 10 ml of 96% ethanol, then it was vacuum filtered through Whatman N° 40 paper, washing the solid once more with ethanol, obtaining 20 ml of final solution. We proceeded to evaluate 1 ml of the solution obtained, proceeding in the same way as the standard, described below. For the calculations, a calibration curve (0 - 13 µg/ml) was made using a 1 mg/ml stock solution of D-fructose as standard, from which seven aliquots were taken, transferred to seven test tubes and mixed. with 1 mL of 5% phenol in water, prepared immediately, and 5 mL of concentrated sulfuric acid, then vortex vigorously for one minute. Once the mixture has cooled to room temperature and in the dark, its absorbance is read at 490 nm to plot µl/ml D-fructose vs. Abs. 490nm. Each sample was tested in triplicate and the results are expressed as milligrams of fructose per gram of leaf dry weight.

2.3.4) SOLUBLE PROTEIN ESTIMATION

• Preparation of necessary reagents:

Potassium phosphate-EDTA buffer for protein extraction: 2.3135 g of $KH_2PO_4$ (MW 136.09 g/mol), 7.53 g of $K_2HPO_4.3H_2O$ (MW 228.23 g/mol), 0.1861g were weighed. of EDTA-$Na_2$ (MW 372.23 g/mol), then the set of reagents was deposited in a beaker and dissolved with 350 ml of cold distilled water. It was stirred until complete dissolution and adjusted to pH 7.5 with 0.1 M HCl, finally making up to 500 ml with cold distilled water. The solution was stored at 4 °C until use.

Sodium phosphate buffer for calibration curve: 1.244g of $NaH_2PO_4.3H_2O$ (MW 173.98 g/mol) and 0.4042 g of $Na_2HPO_4$ (MW 141.96 g/mol) were weighed, the set of reagents were deposited in a beaker and dissolved in 80 mL of cold distilled water, stirring until complete dissolution. It was then adjusted to pH 6.8 with 0.1 M HCl and made up to 100 ml with cold distilled water. The solution was stored at 4 °C until use.

Bradford's reagent: 100 mg of Coomassie Brilliant Blue G-250 were weighed, dissolved in 50 ml of 96% ethanol, shaking vigorously and in the dark, then 100 ml of phosphoric acid 85% were added, also shaking vigorously and in the dark. Subsequently, it was made up to 1.0 l with cold distilled water and stirred for one hour, filtered twice under reduced pressure through Whatman N° 40 filter paper and stored in an amber bottle at 4 ºC.

- Calibration curve:

It was carried out with six different concentration levels with a bovine serum albumin (BSA) standard of concentration 1,45 mg/ml, preparing the reaction mixtures and the procedure presented below: they were placed in six test tubes 0, 20, 40, 60, 80 and 100 µl of stock solution, then 2 ml of sodium phosphate buffer was added, it was homogenized with a vortex for 10 seconds, 800 µL of Bradford's reagent were added and finally each one was covered. The tube and gently homogenized five times to avoid the formation of foam, leaving it to rest for 15 minutes and finally reading the absorbance at 590 nm and 450 nm. Using the sodium phosphate buffer as blank, the graph was constructed: µg/µl protein (X) vs. Abs. 590 nm /Abs. 450 nm (Y).

- Extraction and determination of the content of soluble proteins in the samples:

The extraction was carried out following the method of Deutscher, 1990, with 10 ml of potassium phosphate buffer on 1 g of finely chopped fresh leaf, and homogenizing for two minutes in a vortex. It was incubated for 1 hour in an ice bath on a horizontal shaker and then centrifuged for 30 minutes at 6000 rpm and 4 ºC and the supernatant (protein extract) was separated for immediate determination of protein content. 200 µl of the extract were taken and treated in the same way as the standard in the calibration curve. The results are expressed as milligrams of protein per gram of fresh leaf weight.

2.3.5) PROLINE ESTIMATION

The determination of the amino acid is carried out following the method of Bates et al, 1973.

- Solutions to use:

0.1 M ninhydrin: 156,1 mg of ninhydrin were weighed and dissolved in 3,75 ml of glacial acetic acid at 40 $^0$C, after complete dissolution, 2.5 ml of 3 M phosphoric acid were added.

Sulfosalicylic acid 3%: 1,5 g of sulfosalicylic acid were weighed and dissolved in 50 ml of distilled water, stored at 40 °C.

40 µM proline standard: 2.3 mg of L-proline was weighed and dissolved to 500 ml with the sulfosalicylic acid solution.

- Calibration curve:

It is performed using a previously prepared L-proline standard and evaluating each concentration level in triplicate. The procedure consisted of placing in a test tube with a screw cap covered with aluminum foil, adding the respective volume of 3% sulfosalicylic acid and the different amounts of the standard for each desired point on the curve, then 1 ml of acetic acid was added and 1 ml of the ninhydrin solution, shake vigorously for 10 seconds in a vortex, protecting from light at all times. The test tubes were placed in a rack and brought to a boil in a water bath for one hour, then they were suddenly cooled on an ice-water bath and once they were at room temperature, 3 ml were added to each tube of toluene, shaken vigorously once more and then the organic phase (upper phase) was collected and its absorbance read at 520 nm, using toluene as blank. The graph Proline (µg/ml) vs. Abs 520 nm.

- Extraction and determination of the proline content in the samples:

1 g of fresh chopped leaf from each sample was weighed in 20 ml of 3% sulfosalicylic acid and sonicated for one hour, then centrifuged at 6000 rpm for thirty minutes at 10 °C. 1 ml of the supernatant was taken, then treated in the same way as

 

described in the calibration curve. Analyte content was expressed as micrograms proline per gram of fresh leaf.

2.3.6) GLYCINE BETAINE ESTIMATE

- Calibration curve:

To extract and quantify glycine betaine, the method described by Ma et al (2007). It is performed using a glycine-betaine standard dissolved in 2N $H_2SO_4$ at concentrations ranging from 50 to 200 µg/ml, evaluating each one in triplicate. Each concentration was diluted 1:1 with 2N $H_2SO_4$, then 0,5 mL of this solution was taken, mixed with 0,2 mL of a KI-$I_2$ solution in water, and gently vortexed store at 0 °C for 16 h. Once the 16 h had elapsed, maintaining at 0 $^0$C, we proceeded to centrifuge at 10.000 rpm for 15 minutes and remove the supernatant. The crystals that settled at the bottom of the tube were diluted with 9 ml of 1,2-dichloroethane, mixing vigorously and leaving to settle for 2,5 h to finally read their absorbance at 365 nm. The linear regression line that relates glycine-betaine concentration (µg/ml) vs. Abs. at 365 nm

- Extraction and determination of glycine betaine in the samples:

Both the extraction and the quantification were carried out following the method described by Grieven and Grattan, 1983: 5 g of dry leaf from each sample, from each one of the plants, were placed to macerate in 10 ml of 2N $H_2SO_4$ for 48 h in the dark. . We proceed to evaluate 1 ml of the supernatant, in the same way that was done for the standard. The result are expressed as micrograms of glycine-betaine per gram of leaf dry weight.

2.4) STATISTICAL ANALYSIS

ANOVA comparisons were made using the statistical program STATGRAPHICS 2.0, to evaluate the existence of significant differences both between the healthy plant and the diseased plants, as well as between the diseased plants. Data with $P \leq 0.05$ were considered statistically significant. All assays were performed in triplicate for each plant.

## 4. Results and Discussion

The abbreviations used are clarified below:

PS: plant that did not show viral titer (used as negative control)
PE1V: plant infected only with GLRaV-3 (one viruse)
PE2V: plant infected with GLRaV-3 coinfected with and GLRaV-2 (two viruses)
PE3V: plant infected with GLRaV-2 coinfected with GLRaV-3 and GVA (three viruses)

3.1) VIRAL DETECTION AND IDENTIFICATION

One plant was reported without identifiable virus, so it was taken as a control plant (negative control), another three plants were identified as plants infected with viral pathogens, one with GLRaV-3, another coinfected with GLRaV-3 and GVA, and one third infected with GLRaV-2, GLRaV-3 and GVA (positive controls, one virus, two viruses and three viruses respectively)

3.2) ESTIMATION OF α-TOCOPHEROL AND ANTIOXIDANT COMPOUNDS.

It was observed that the plants infected with one, two and three viruses (PE1V, PE2V and PE3V) presented, with respect to the healthy plant (PS), an average increase in α-tocopherol of 50, 53.57 and 89.28 % respectively (Table I), representing, in global terms, the infected plants an average increase of 64.28% with respect to the control plant. The $EC_{50}$ was decreased with respect to the healthy plant by 24.03 % for PE1V, by 22.92 % for PE2V and by 26.74 % for PE3V, thus demonstrating that the amount of antioxidant compounds present in the samples of diseased plants was greater than in the healthy plant, in global terms this increase represented 24.56 % (Table I).

Tocopherol plays an important role as an [23]. Tocopherol is located in some biological membranes, especially in chloroplasts and thylakoids, where its main, and sometimes only, function is to actively regulate lipid radicals and reactive oxygen species**.** Of the four isomers present in nature (α, β, γ and δ), α-tocopherol is the one with the highest antioxidant activity, due to the three methyl groups present in its structure. α-tocopherol has an important role in the protection of cell membranes against ROS [22]. So far, numerous investigations have shown that the amount of α-tocopherol increases during stress [33]. This compound and its isomers readily react with free lipid radicals and protect against ROS formed during stress. When plants are exposed to a pathogen, the formation of ROS occurs mainly in the leaves, causing an accumulation of antioxidant compounds in them. α-Tocopherol, it cooperates to maintain the structure of cell membranes, in order to avoid the oxidation of unsaturated fatty acids, caused by these chemical compounds [24]. The increase in α-tocopherol in tissues of plants infected with the viruses under study suggests that it can improve resistance to them, protecting cell membranes from oxidative damage caused by stress, and also indicates a greater ability to adapt on the part of plants to viral infection. The increase in the synthesis of α-tocopherol and its subsequent accumulation in cells is consistent with previous research, stating that this metabolite is generated in response to the biotic stress that plants are suffering [58], [60].

Phenolic compounds, classified as antioxidant compounds, are secondary metabolites capable of reacting against ROS, thanks to the ability of the phenolic hydroxyl(s) to donate hydrogen [37]. The production of these by the cell is affected by different types of stress: biotic stress such as attack by pathogens [28], or abiotic (hydric stress, saline stress, etc.), which can increase while the stress lasts [5][7]. Phenolic compounds may or may not accumulate, depending on whether the interaction between silver and the pathogen is compatible or not, which will develop or not the Hypersensitive Response, increasing both the amount of these substances and the enzymes involved in their biosynthetic pathways [69]. From the point of view of plant defenses against different pathogens, the detoxification of ROS and its generating enzymes must be maintained at a threshold that is compatible with the cellular metabolism itself, and does not

 

negatively affect it when reaching a point of apoptosis [56][12].

Under optimal conditions, ROS are produced by many metabolic reactions and are efficiently eliminated by detoxification processes. Under stress conditions there is an increase in ROS, and as a consequence an increase in detoxifying enzymes and compounds with the ability to neutralize them, as a mechanism of "acclimatization" (upregulation of ROS and detoxification capacity) or induction of death cell phone [34], [39]. The decisive factors that determine either acclimatization or cell death are not yet known. It has been shown that plant cellular metabolism is capable of forming ROS and increasing the activity of enzymes involved in its metabolic pathways in response to both compatible and incompatible infections following pathogen attack. [16]. Investigations have reported the increase of phenolic compounds against plant pathogenic fungal, bacterial and viral interactions, such as *in Solanum lycospersicum* plants infected with *Ralstonia solanacearum* [74] and *Musa paradisica* L. plants infected with *Colletotrichum musae* [20]. The results of the present investigation are consistent with what was reported by Zaho et al (2008), where an increase in antioxidant activity is demonstrated, and consecutively, an increase in the amount of phenolic compounds, in plants infected with GLRaV-2, GLRaV -3 and GVA, which suggests the ability of the plant to defend itself from the pathogen, once the interaction has been compatible.

### 3.3) ESTIMATION OF SOLUBLE SUGARS AND SOLUBLE PROTEINS.

The soluble sugar content of diseased plants (PE1V, PE2V and PE3V) was increased by 23,33, 23,34 and 24,22 % for diseased plants with one, two and three viruses respectively compared to healthy plant (Table I). The average increase of sugars for the diseased plants with respect to the healthy plant represented 23,59 %. It was observed that the plants infected with one (PE1V), two (PE2V) and three (PE3V) viruses presented an increase in soluble proteins, expressed as mg of BSA per fresh leaf weight, of 24,55 %, 15,29 % and 19,53 % with respect to the healthy plant (Table II). This in global terms represents that the diseased plants present an average increase of 19,79 % with respect to the control plant (PS). An increase in the soluble sugar content could be attributed to the denaturing effect against the lipoprotein complexes that degrade during biotic stress [36]. The results of this study are consistent with others that showed, for example, that infection with *Sugarcane yellow leaf virus* (ScYLV) resulted in a notable increase in the amount of soluble carbohydrates and starch in the leaves of sugarcane plants [45], [27], [41]. Carbohydrate accumulation has also been demonstrated in potato plants (*Solanum tuberosum*) infected with *Potato leafroll virus* [68]. The increase in soluble sugars in symptomatic and asymptomatic leaves of plants with viral infection could be a product of virus assembly in the phloem, and as a consequence of a violation of the virus function per se, altering the origin/destination relationship of sugars synthesized, since these could not be correctly transported to the fruits [42].

Some investigations have shown that plants infected with GLV have high levels of osmotic stress, And this significantly influences the amount of soluble proteins synthesized and accumulated in the different parts of the plant. [14], [17]. For example, a study on plants infected with GFLV and GLRaV-3 indicated an increase in total protein content in grapevine plants infected with GLV [66], [9], [14]. Increased soluble protein content has also been reported in BBTV-infected banana plants [31] and in PLRV or PVY-infected potato plants [43]. According to Bertamini et al (2009), a pronounced reduction in the amount of soluble proteins in grapevine A decrease in the production of ribulose-1,5-bisphosphate carboxylase is assumed. It is then understood that an increase in protein synthesis, whether soluble or non-soluble, is due to an increase in general protein synthesis in response to oxidative stress that is caused by viral infection **[**61]. The results of the present investigation are consistent with previous investigations, demonstrating that infection with GVA and with GLARaV It triggers an increase in soluble protein synthesis pathways in *Vitis vinifera* plants.

Table 1: α tocopherol content, antioxidant compounds and soluble sugars in the healthy plant and diseased plants.

| Plant | α-tocopherol (µg/g FW) | Antiox. Comp., $EC_{50}$ (µg/ml FW) | Soluble sugars (mg/g DW) |
|---|---|---|---|
| PS | 541,3 ± 0,23 | 11,93 ± 0,31 | 21,44 ± 0,8 |
| PE1V | 812 ± 0,00 | 9,06 ± 0,98 | 26,42 ± 0,6 |
| PE2V | 831,3 ± 0,03 | 9,19 ± 1,45 | 26,42 ± 0,2 |
| PE3V | 1024,7 ± 0,03 | 8,74 ± 0,83 | 26,63 ± 1,2 |
| p values | | | |
| PS/PE1V | *** | *** | ** |
| PS/PE2V | *** | *** | ** |
| PS/PE3V | *** | ** | *** |
| PE1V/PE2V | ns | ns | ns |
| PE1V/PE3V | ns | ns | ns |
| PE2V/PE3V | ns | ns | ns |

Note: *** - $p < 0,001$, ** - $p < 0,01$, * - $p < 0,05$, ns – no significative. FW: fresh weight, DW: dry weight.

### 3.4) PROLINE AND GLYCINE-BETAINE ESTIMATION.

The proline content of diseased plants was increased by 40,55, 50,85 and 41,38 % for diseased plants with one, two and three viruses respectively compared to healthy plant (Table II). The average increase of proline with respect to the healthy plant represented 44,26 % more. In diseased plants with one virus, two and three, the glycine-betaine content was increased by 72,50 %, 45,43 % and 57,68 % respectively, compared to the healthy plant, used as a negative control plant (Table II). The average increase of diseased plants was 58,54 % with respect to the healthy plant.

In the present investigation, a high amount of proline in plants infected with GLRaV and GVA was observed in comparison with healthy plants (control plants). The results presented here are consistent with those of similar

 

investigations [21]. For example, it was found that in potato plants infected with *Potato virus Y* and *Potato virus X*, proline concentrations increased a few days after infection [11]. This was also observed in "soybean" plants (*Glicine max*) under saline stress [32], or also in rice plants (*Oryza sativa* L.) [67]. Microbial pathogens promote ROS production, which can induce programmed cell death in some leaf and stem cells [5] [1]. The proline molecule functions as a preventer of apoptosis, which is triggered by ROS, fulfilling the function of both cytoprotector and osmoprotector [15]. Thus, the accumulation of this primary metabolite is explained as a product of GLRaV and GVA infection [21]. The glycine betaine molecule is a product of glycine metabolism; both have a prominent role in the activation of membrane phospholipid synthesis pathways [6]. The protein holin and two enzymes, holinmonooxygenase and betaineadialdehyde dehydrogenase, play an important role in the synthesis of the glycine betaine molecule in plants [26]. Glycine betaine plays the role of osmolyte and osmoprotect, accumulating when the plant is under some type of stress, thus becoming involved in its protection through the regulation of the osmotic balance [63]. The stabilization of antioxidant enzymes and the structure of membrane proteins belonging to the complexes of oxygenic photosynthesis of photosystem II [62]. The glycine betaine molecule is responsible for regulating the photosynthetic function of the plant, thus protecting cells from damage caused by different types of stress, fulfilling the function of promoter of the enzyme ribulose-1,5-bisphosphate carboxylase. oxygenase (RuBisCO) [65]. Under the assumptions explained above, we can infer that the synthesis and subsequent accumulation of the glycine betaine molecule in the leaves of infected plants occurs as a consequence of the viral infection, which especially affects the phloem, not allowing the correct transport of nutrients towards the cells of the leaves, tending these molecules to protect cell membranes from damage caused by stress. The results of this thesis are consistent with these investigations. The infection by GVA, GLARaV-1, GLARaV-2 and GLARaV-3, causes an increase in the synthesis of glycine betaine and its subsequent accumulation in leaves.

Table 2: content of soluble proteins, proline and glicine betaine in healthy plant and diseased plants.

| Plant | Soluble proteins (mg/g FW) | Proline (mg/g DW) | Glycine betaine (µg/g DW) |
|---|---|---|---|
| PS | 71,08 ± 7,73 | 2,58 ± 0,42 | 18,48 ± 1,52 |
| PE1V | 88,73 ± 6,53 | 3,63 ± 0,19 | 31,88 ± 2,68 |
| PE2V | 81,95 ± 1,95 | 3,90 ± 0,29 | 26,88 ± 3,02 |
| PE3V | 85,17 ± 8,48 | 3,65 ± 41,38 | 29,14 ± 2,41 |
| p values | | | |
| PS/PE1V | *** | *** | *** |
| PS/PE2V | ** | *** | ** |
| PS/PE3V | *** | *** | *** |
| PE1V/PE2V | ns | ns | * |
| PE1V/PE3V | ns | ns | ns |

Note: *** - p < 0,001, ** - p < 0,01, * - p < 0,05, ns – no significative. FW: fresh weight, DW: dry weight.

## 6. Conclusion and Future Scope

In the present investigation it was determined that, under viral infection by *Leafroll virus* and *Grapevine Virus A*, *V. vinifera* plants respond by increasing the concentration of some secondary and primary metabolites, such as proline, glycine betaine, proteins and sugars, which have some function specific protection against stress. These results are also consistent with previous research on the subject [57], [58], assuming that any of these metabolites studied could result, as explained above, as a chemical elictor when these plants are infected with the aforementioned pathogens, being eco-friendly chemical compounds, not synthetics. The results of this research serve as a basis for future research in order to search for new methods for the early detection of vine curling, avoiding its rapid spread, and thus, as a consequence, the great losses to fruit producers.

### Data Availability

All data obtained as results of this research are available in this document. There is no hidden or unavailable data. There is no limitation that conditions this research.

### Conflict of Interest

All authors declare that there are no declared or potential conflicts of interest.

### Founding source

None

### Author´s contribution

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