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RAMAN spectroscopy applications in grapevine: metabolic analysis of plants infected by two different viruses

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Author contribution statement

LM, CD, SM, FN, GB performed the experiments. LM, EN, GB, GG, AMG, FN analyzed data. LM, GG, and EN wrote the manuscript. LM, GG, AMR, and EN conceived the study and participated in its design. All the authors contributed to the article and approved the submitted version.

Keywords

Raman Scattering, *Vitis vinifera*, Carotenoids, virus, early diagnosis

Abstract

Word count: 233

Grapevine is one of the most cultivated fruit plant among economically relevant species in the world. It is vegetatively propagated and can be attacked by more than 80 viruses with possible detrimental effects on crop yield and wine quality. Preventive measures relying on extensive and robust diagnosis are fundamental to guarantee the use of virus-free grapevine plants and to manage its diseases. New phenotyping techniques for non-invasive identification of biochemical changes occurring during virus infection can be used for rapid diagnostic purposes. Here, we have investigated the potential of Raman spectroscopy (RS) to identify the presence of two different viruses, grapevine fan leaf virus (GFLV) and grapevine rupestris stem pitting-associated virus (GRSPaV) in *Vitis vinifera* cv. Chardonnay. We showed that RS can discriminate healthy plants from those infected by each of the two viruses, even in the absence of visible symptoms, with accuracy up to 100 and 80% for GFLV and GRSPaV, respectively. Chemometric analyses of the Raman spectra followed by chemical measurements showed that RS could probe a decrease in the carotenoid content in infected leaves, more profoundly altered by GFLV infection. Transcriptional analysis of genes involved in the carotenoid pathway confirmed that this biosynthetic process is altered during infection. These results indicate that RS is a cutting-edge alternative for a real-time dynamic monitoring of pathogens in grapevine plants and can be useful for studying the metabolic changes ensuing from plant stresses.

Contribution to the field

New phenotyping techniques for non-invasive and rapid identification of plant pathogens are critical to prevent the spread of the disease, contributing to reduce the economic damages. Raman spectroscopy (RS) is being proposed as a cutting-edge technology for this purpose. Here, we have applied RS on grapevine, one of the most worldwide cultivated and economically relevant fruit crop. Grapevine can be attacked by a huge number of pathogens, including viruses, pushing to implement extensive and robust diagnostic programs to guarantee the use of virus-free plants. We show that RS can discriminate healthy plants from individuals infected by two different viruses, with high levels of accuracy and even in the absence of visible symptoms. Chemometric analyses of the Raman spectra followed by chemical measurements showed that RS probed a decrease in the carotenoid content in infected leaves. Transcriptional analysis of genes involved in the carotenoid pathway confirmed that this biosynthetic process is altered during infection. Therefore, RS is a suitable tool to monitor in real-time the dynamics of pathogen infection in grapevine plants and it is useful for studying the metabolic changes ensuing from plant stresses.

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In review

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3 **plants infected by two different viruses**

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18 **Keywords: Raman scattering, *Vitis vinifera*, carotenoids, early diagnosis, virus**

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20
21 **ABSTRACT**

22 Grapevine is one of the most cultivated fruit plant among economically relevant species in the
23 world. It is vegetatively propagated and can be attacked by more than 80 viruses with possible
24 detrimental effects on crop yield and wine quality. Preventive measures relying on extensive and
25 robust diagnosis are fundamental to guarantee the use of virus-free grapevine plants and to manage
26 its diseases. New phenotyping techniques for non-invasive identification of biochemical changes
27 occurring during virus infection can be used for rapid diagnostic purposes. Here, we have
28 investigated the potential of Raman spectroscopy (RS) to identify the presence of two different
29 viruses, grapevine fan leaf virus (GFLV) and grapevine rupestris stem pitting-associated virus
30 (GRSPaV) in *Vitis vinifera* cv. Chardonnay. We showed that RS can discriminate healthy plants
31 from those infected by each of the two viruses, even in the absence of visible symptoms, with
32 accuracy up to 100 and 80% for GFLV and GRSPaV, respectively. Chemometric analyses of the
33 Raman spectra followed by chemical measurements showed that RS could probe a decrease in the
34 carotenoid content in infected leaves, more profoundly altered by GFLV infection. Transcriptional
35 analysis of genes involved in the carotenoid pathway confirmed that this biosynthetic process is
36 altered during infection. These results indicate that RS is a cutting-edge alternative for a real-time
37 dynamic monitoring of pathogens in grapevine plants and can be useful for studying the metabolic
38 changes ensuing from plant stresses.

39 INTRODUCTION

40 Grapevine (*Vitis vinifera* L.) is one of the most important fruit crop, with up to 7 million hectares
41 cultivated worldwide in 2020 (FAOSTAT, 2020). Grapevine is mainly grown for wine production
42 and for fresh and dry fruit consumption, but it is also used for seed oil extraction, alcoholic
43 beverage and vinegar production; moreover, several social, touristic and cultural activities are
44 linked to its cultivation, generating a positive impact on the economy.

45 Grapevine is affected by several pathogens, including fungi, oomycota, phytoplasmas, and viruses
46 heavily influencing yield and quality of the crop and reducing the economic revenues. Among
47 grapevine pathogens, viruses are widespread in all cultivated areas, causing different diseases, such
48 as the rugose wood complex, leafroll, infectious degeneration, and fleck disease (Fuchs, 2020). Up
49 to now, more than 80 viruses from 17 families and 34 genera have been identified (Martelli et al.,
50 2014, 2018), frequently occurring in mixed infection.

51 Within this large number of viral entities threatening grapevine, grapevine rupestris stem pitting-
52 associated virus (GRSPaV) and grapevine fanleaf virus (GFLV) are two well-known and
53 widespread examples. After its discovery about two decades ago, GRSPaV is nowadays considered
54 one of the most ubiquitous viruses, found in Europe, America, Australia, and Asia (Meng and
55 Rowhani, 2009). GRSPaV belongs to the genus *Foveavirus*, family *Betaflexiviridae*, and it is
56 generally associated to “Rupestris Stem Pitting”, a disorder of the “Rugose Wood complex” (Meng
57 and Gonsalves, 2003). Its presence has been linked to other grapevine diseases, including the vein-
58 clearing complex on cv. Chardonnay (Lunden et al., 2009). Nonetheless, in most cases GRSPaV
59 induces latent infections, with no visible symptoms on infected plants. Despite this, GRSPaV was
60 reported to trigger a number of transcriptional changes on cv. Bosco, mainly regarding
61 photosynthesis and CO₂ fixation, leading to a moderate decrease of the photosynthetic process and
62 an altered reaction of plants to biotic/abiotic stress, underlying possible beneficial effects mediated
63 by this virus towards abiotic factors (Gambino et al., 2012; Pantaleo et al., 2016; Tobar et al, 2020).

64 GFLV (family *Secoviridae*, genus *Nepovirus*) is a harmful and economically deleterious virus,
65 responsible for the ‘Grapevine infectious degeneration’ complex (Sanfacon et al., 2009). Symptoms
66 induced by GFLV include vein yellowing, mosaics, internode shortening, typical leaf deformations,
67 smaller and fewer bunches, with irregular ripening. The variability of symptoms observed in
68 vineyards depends on the virus strain, grapevine genotype, cultural practices, and environmental
69 conditions (Martelli, 2017). GFLV is transmitted by the soil-borne ectoparasitic nematode
70 *Xiphinema index* and by infected plant material. Beside phenotypic alterations typical of infectious
71 degeneration, the physiological and molecular changes induced by GFLV can be occasionally
72 associated to an improved tolerance towards fungal infections (Gilardi et al., 2020) and to a
73 moderate water stress (Krebelj et al., 2022). Overall, GRSPaV and GFLV represent two virus
74 models regarding the symptomatology induced on vine plants, that interact with the host in complex
75 and unexpected ways, justifying to more deeply explore the changes occurring during the infection
76 processes.

77 Early diagnosis of plant pathogens is crucial for a proper disease management, allowing not only to
78 eliminate infected material and reduce further spread of the pathogens, but also to implement clean
79 stock programs useful to preserve the sanitary status of a crop. This is particularly relevant for
80 grapevine, a vegetatively propagated perennial crop, and for viral pathogens which cannot be
81 eliminated with chemical pesticides. For these, in fact, eradication programs are required before the
82 nursery stage and during the clonal selection, currently performed applying sanitation techniques
83 such as meristem culture, thermotherapy, and somatic embryogenesis. Specifically, due to the

84 extensive use of clonal multiplication of grapevine, many countries have established strict
85 regulations for the grapevine propagation material, in order to verify the presence of viruses and
86 reduce the risk of disease spread (Golino et al., 2017). Plant disease diagnosis is commonly
87 performed using molecular-based procedures (Fang and Ramasamy, 2015; Martinelli et al., 2015),
88 which can be time consuming, unsuitable for rapidly testing large numbers of samples, require
89 skilled personnel and the availability of pathogen-specific reagents (gene sequences or antibodies),
90 and are not frequently implemented for field application. Indeed, grapevine certification schemes
91 mainly rely on serological and molecular assays, aided by biological indexing, time consuming and
92 expensive activities often requiring multiple evaluations. In Italy, sanitary schemes dictate that all
93 materials test negative for grapevine virus A (GVA), GFLV, Arabis mosaic virus (ArMV),
94 grapevine leafroll-associated virus-1 and -3 (GLRaV-1, -3), and grapevine fleck virus (GFkV, this
95 only for rootstocks) (Italian regulation D.M. 7/07/2006 and D.L. 02/02/2021). Therefore, new
96 diagnostic tools, ideally suitable for field testing of plants by untrained personnel, using friendly
97 and inexpensive equipment and providing results in a short time, with minimal number of steps
98 would be extremely important. Such strategies could allow extensive and fast screening of imported
99 vegetative material, preventing disease spread.

100 Raman spectroscopy (RS) records the molecular vibrations of cellular metabolites present in a
101 specimen in the absence of labels or reagents and has been recently proposed as a non-destructive
102 and rapid diagnostic procedure for plant pathogens. The spectra obtained from healthy and diseased
103 plant samples are used as specific fingerprints, reflecting changes in cellular metabolites occurring
104 following infection by pathogens or during abiotic stresses. Indeed, several groups including our
105 laboratory have shown that RS can sense the presence of different plant pathogens, among which
106 viruses, in different cultivated crops (Yeturu et al., 2016; Egging et al., 2018; Farber and Kurouski,
107 2018; Farber et al., 2018, 2019a,b; Sanchez et al., 2020). In particular, we showed that specific
108 changes in tomato plants artificially inoculated with two different viruses can be identified by RS, at
109 a stage when visual symptoms were not yet visible (Mandrile et al., 2019).

110 In the current study, we investigated the potential of RS to determine the occurrence of two
111 different viruses infecting grapevine cv. Chardonnay; the two pathogens were chosen as examples
112 of a latent-asymptomatic virus (GRSPaV) and a dangerous-symptomatic virus (GFLV), whose
113 absence is required in the certification protocols. Plants separately infected by the two viruses were
114 analyzed with a Raman microscope apparatus at different time points during the vegetative season
115 and systemic molecular changes induced by the viruses were analyzed by RT-qPCR.

116 MATERIAL AND METHODS

117 Plants

118 *V. vinifera* cv. Chardonnay plants infected by either GFLV (cluster IB) (NCBI Acc. No.
119 MN889891) or GRSPaV (phylogenetic group GRSPaV-SG1) (NCBI Acc. No. MN889892) were
120 previously described in Gilardi et al. (2020). In this work, two years-old infected plantlets and
121 healthy individuals (n=4) were maintained in 5-liter pots filled with a peat substrate (TS4, Turco
122 Silvestro, Italy). Plants were kept under a gauze greenhouse for the whole duration of the
123 experiment, with constant watering. Each plant represents a biological replica.

124 RNA Extraction and RT-qPCR

125 Total RNA was extracted using a rapid CTAB method (Gambino et al., 2008) and its quantity and
126 quality were evaluated with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific,
127 Waltham, MA, USA). RNA was then treated with DNase (DNase I, Thermo Fisher Scientific,

128 Waltham, MA, USA) and reverse-transcribed using the High-Capacity cDNA Reverse
129 Transcription Kit (Thermo Fisher Scientific), following manufacturer's instructions.

130 RT-qPCR reactions were performed in a CFX Connect Real-Time PCR system (Bio-Rad
131 Laboratories, Hercules, CA, USA), using SYBR Green (SensiFAST™ SYBR® No-ROX Kit;
132 Meridian Bioscience, Memphis, Tennessee, USA) with the following cycling conditions:
133 denaturation at 95°C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. RT-
134 qPCR was conducted for the relative quantification of GFLV and GRSPaV, using primers specific
135 for viral RdRp (Gilardi et al., 2020), and for transcriptional analysis of genes representative of the
136 carotenoid pathway, using Ubiquitin (*VvUBI*) and Actin1 (*VvACT1*) as internal controls. The
137 primers for RT-qPCR are listed in Table S1. Four independent biological replicates and three
138 technical replicates were run for each RT-qPCR. Gene expression data were subjected to analysis of
139 variance (ANOVA), followed by the Tukey's HSD post hoc test ($p \leq 0.05$). The SPSS statistical
140 software package (SPSS Inc., Cary, NC, United States, v.23) was used to run statistical analyses.

141 **Raman Spectroscopic Measurements**

142 Raman spectra were acquired from one half of the fifth leaf counting from the apex, while the other
143 half was used for virus detection and transcript accumulation analysis. Leaf samples for RS analysis
144 were stored in plastic bags and kept on ice until spectra acquisition within the following 4 h. Spectra
145 ($400\text{--}3100\text{ cm}^{-1}$; 5 cm^{-1} resolution) were acquired using a Dispersive Raman Spectrometer (DRX
146 Thermo Fisher Scientific, Waltham, U.S.A.; 785 nm excitation laser, 10× microscope objective, 2
147 μm laser spot diameter, 10 mW laser power; 20 scans, 1 sec each, were collected on the same
148 point of the leaf, taking three points per leaf, on three different leaf lobes.

149 The spectrometer was weekly calibrated using a certified white light for intensity and neon gas lines
150 for frequency. Moreover, a Si standard was measured before each session, to guarantee consistency
151 within measurements and to avoid differences due to instrument performances. Four different
152 measurements were performed, at monthly intervals, starting in May, until August 2021 (T1 to T4).

153 **Chemometric Analysis of Raman Spectra**

154 Chemometric analysis was conducted using the PLS Toolbox (Eigenvector Research, Inc., Manson,
155 WA) for Matlab R2015a (Mathworks, Natick, MA). Spectral range between $650\text{--}3060\text{ cm}^{-1}$ was
156 considered. Spectra pre-processing consisted in smoothing (Savitzky-Glay filter, 21 pt.), baseline
157 correction (automatic weighted least square regression, 2nd order and Whittaker filter with
158 asymmetry $1e^{-5}$, $\lambda 1000$), and mean centering. Principal Component Analysis (PCA) was used to
159 find non-random data structures attesting non-random variability between groups of spectra. The
160 effect of the different factors of the experimental design was evaluated by analysis of variance
161 simultaneous component analysis (ANOVA-SCA, also known as ASCA), considering the following
162 k factors: (i) "time" (T1, T2, T3, T4); (ii) "virus" (presence of infection; levels healthy, GRSPaV
163 and GFLV, and (iii) "biological replicates" (levels: different plant specimens).

164 ASCA was performed considering the two-way correlations between factors. The significance of
165 the experimental factors was quantified determining p-values through a permutation test between
166 the levels of the factors (Zwanenburg et al., 2011). The H0 hypothesis of no experimental effect,
167 indicating no difference between the levels averages of the effect matrices, with a confidence level
168 of p was tested. P-values were obtained for the main effects by randomizing the levels of each
169 factor under consideration.

170 PLS-DA was finally used as a classification method to test the possibility to recognize infected
171 plants. Since an external test set for validation was not available, leave-one plant-out cross
172 validation (CV) was used to determine the classification error (CE).

173 **Sample extraction and analysis of total carotenoids, chlorophylls, and polyphenols**

174 Plant extracts were prepared according to Alrifai et al. (2021), with slight modifications. Grapevine
175 leaves were freeze-dried and maintained at -80 °C; 25-30 mg of powdered material were extracted
176 in 4 ml of an acetone:ethanol (1:1, v/v) solution and extracts were sonicated in a water bath for 15
177 min and incubated at room temperature for 4 h, with shaking at 400 rpm (Sky4 Shaking Incubator,
178 Argo Lab). After centrifugation (1600 × g, 5 min), each supernatant was transferred to a clean tube;
179 pellets were re-extracted twice with the same solvent, once using 2 ml for 2 h, followed by 1 ml for
180 1 h. Supernatants from the same sample were pooled. For total carotenoid and chlorophyll content
181 analysis, a 250-µl aliquot of each extract was added in triplicate to a 96-well microplate. The plate
182 was analyzed immediately using a UV/VIS Varioskan Lux (Thermo Fisher Scientific, Waltham
183 USA) multi-wells reader, measuring absorbance at 452 nm. A calibration curve was prepared with a
184 β-carotene (Sigma Aldrich, Certified Reference Material, >99%) solution, using at least five
185 concentrations from 2 µg/ml to 50 µg/ml, $R^2 > 0.99$. Total carotenoid content was expressed as µg
186 of β-carotene equivalents/g of dry weight sample. Absorbance at 666 nm was also recorded to
187 evaluate the chlorophyll content and relative comparison between the tested samples was performed
188 to provide semi-quantitative information.

189 Total polyphenol content was measured by the Folin-Ciocalteu method, using the same
190 ethanol:acetone (1:1) leaf extracts (see above). Aliquots of 200 µl of each extract were added to 15-
191 ml tubes containing 3 ml ultrapure water and 200 µl Folin-Ciocalteu reagent (Sigma Aldrich). After
192 mixing and incubating the samples for 6 min at room temperature, 200 µl of 20% (w/v) Na₂CO₃
193 (Carlo Erba) were added to each tube and vortexed. After 30 min incubation at 37 °C, aliquots of
194 200 µl of each sample were placed in triplicate in a 96-wells microplate and absorbance at 765 nm
195 was measured with the UV/VIS Varioskan Lux multi-wells reader, by subtracting the absorbance of
196 the blank (ethanol:acetone solution, 1:1). A calibration curve made with gallic acid was used as
197 standard, measuring at least five concentrations from 40 to 200 mg/l. Results were normalized to
198 the weight of the dried leaf sample (mg/l).

199 **EXPERIMENTAL RESULTS AND DISCUSSION**

200 **Virus accumulation in grapevine plants along the vegetative season**

201 In this work, we considered grapevine plants cv. Chardonnay infected by either GRSPaV or GFLV,
202 and healthy control individuals (Gilardi et al., 2020). Plants were surveyed along the whole
203 vegetative season from May to August 2021, at four different time points (T1 to T4) at monthly
204 intervals. During the whole season, no visible symptoms could be detected on plants infected by
205 either virus, in agreement with a previous report (Gilardi et al., 2020) and in line with unpublished
206 observations of young plants kept in pots, across several years (G. Gambino, personal
207 observations).

208 RT-qPCR virus quantification analysis showed an overall stable accumulation of GRSPaV along
209 the whole duration of the experiment, with a slight increase only at the end of the season (Figure 1).
210 In vineyard conditions, the GRSPaV titer in leaves tends to decrease as the season progresses, while
211 no such decrease occurred in the present conditions (Gambino et al., 2012). On the contrary, a
212 remarkable drop in the accumulation of GFLV occurred since the second time point analyzed (T2,
213 June), with no further changes during the vegetative season (Figure 1). The reduction of the GFLV

214 titer along the season is in line with observations recorded in vineyard, where the highest GFLV
215 concentrations in leaves were found in May, i.e. at the beginning of the vegetative period (Krebelj
216 et al., 2015, Gilardi et al., 2020).

217 **Raman spectra measurements of leaves**

218 The Raman spectra of grapevine leaves were collected on intact plant material, focusing the excitation
219 laser directly onto the leaf surface. A near infrared laser wavelength was used to limit the undesired
220 fluorescence effect disturbing Raman signals. Other research paper dealing with Raman
221 measurements on plant tissues report the alternative use of 785 nm (Dou et al., 2021), 830 nm (Farber
222 et al., 2019b; Sanchez et al., 2020; Payne et al., 2022), or 1064 nm (Yeturu et al., 2016; Farber and
223 Kourowsky, 2018; Skoczowski et al, 2022) laser wavelengths to minimize fluorescence interference
224 and increase signal to noise ratio. At the same time, a relatively low laser power and low
225 magnification objective were adopted to avoid thermal stress of the tissue and to collect information
226 from a relatively large area (spot size > 2 μm). The mean spectra of grapevine leaves showed
227 vibrational bands that were assigned to cellulose, carotenoids, polyphenols, chlorophylls, xylan,
228 lignin, and proteins, being the major components of leaves (Figure 2). The assignment of bands of
229 the most relevant peaks are reported in Table 1. According to previous literature, most of the
230 wavenumbers were related to photosynthetic pigments (Zeng et al., 2021).

231 Following this analysis, the spectra obtained from healthy plants were compared with those
232 collected from virus-infected plants, at the different time points. Similar spectral profiles were
233 registered among the three different groups of samples, at the different time measurements (Figure
234 2), indicating that, at preliminary observation, the spectral fingerprint of leaves was not severely
235 influenced by the presence of virus infection, **but only minimal changes were registered. The entire
236 fingerprint regions of the mean spectra for the three classes of plants and the four sampling times
237 are shown in supporting information (Figure S1) for a better comparison. For the majority of bands,
238 frequency mismatches between healthy and infected plants can be noticed since the third sampling
239 time.**

240 Previous works have determined the assignment of Raman bands obtained from leaf samples which
241 are mostly due to carotenoids, being among the most Raman active classes of compounds present in
242 such tissue (Yeturu et al., 2016). In particular, the most evident peak observed at 1526 cm^{-1} is
243 assigned to the stretching of the $-\text{C}=\text{C}-$ double bond in the conjugated chain of carotenoids (Adar,
244 2017), while the shoulder at 1550 cm^{-1} is due to chlorophylls. Focusing our attention on this
245 particular band and comparing the mean spectra of healthy and infected plants monitored during the
246 entire vegetative season, a reduced carotenoid concentration in leaves of GFLV-infected plants was
247 noticed since the second measurement (T2). On the contrary, no such tendency occurred in healthy
248 plants or in GRSPaV-infected plants (Figure 3). In addition, a frequency change that exceeds the
249 resolution limit of 5 cm^{-1} , was registered **in infected tissues for the carotenoid peak, as well as for
250 other bands in the Raman fingerprint region, since the second sampling** (Figure 3). In particular, the
251 $-\text{C}=\text{C}-$ stretching shifted to a slightly lower frequency in infected plants (from 1526 cm^{-1} to 1518
252 cm^{-1} for GFLV and from 1526 cm^{-1} to 1520 cm^{-1} for GRSPaV, at T3), possibly resulting from a
253 modification of the carotenoids profile occurring in these plants (Figure 3). A previous study by
254 Withnall et al (2003) showed a linear inverse dependency of the frequency location of the band of $-\text{C}=\text{C}-$
255 double bonds and the length of the conjugated chain of carotenoids. **However, due to the
256 intrinsic limits of Raman measurements on complex biological matrices, the available data do not
257 allow to specifically address the accumulation of carotenoid molecules of a specific length, an issue
258 which should be investigated with more selective techniques.**

259 Overall, the modification of the Raman peaks, especially those associated to carotenoids, provides
260 an indication that the infection by these two viruses leads to a different metabolic response of
261 infected plants. In particular, a reduced concentration of carotenoids in grapevine suggests a
262 functional link to either a modulation of transcripts involved in carotenoid metabolism or to their
263 degradation and fragmentation or conversion to apocarotenoids, i.e. signaling molecules produced
264 in response to stress. A decrease in carotenoid concentration has been frequently reported when
265 analyzing by Raman spectroscopy plants infected by pathogens (Dou et al., 2021; Farber et al.,
266 2021; Vallejo-Pérez et al., 2021) or subjected to abiotic stresses (Altangerel et al., 2017; Sng et al.,
267 2020), confirming the role of this class of molecules in plant stress responses.

268 Beside the visual comparison of the average spectra collected from healthy and infected plants over
269 time, a more complete investigation regarding the changes in the Raman profiles was conducted,
270 with a multivariate unsupervised visualization method. This procedure allows to consider the whole
271 spectral information and to test the significance of spectral differences within the groups included in
272 the experimental design. For this, the entire dataset was processed with ASCA using the four plants
273 present in each group (factor 'Infection', levels 'healthy', 'GRSPaV', 'GFLV'), considering one
274 leaf per plant, three spectra per leaf, four sampling sessions over four measurements, at monthly
275 intervals (factor 'Time', levels 'T1', 'T2', 'T3', 'T4'). This process is expected to model the effect
276 of each of the factors included in the experimental design and to evaluate the significance of each
277 effect. At the same time, a PCA model was calculated for each design factor, to help visualizing the
278 results. Then, the significance of each factor was tested by permutation tests within the levels of the
279 factors, providing a $p < 0.5$ value for significant factors. Unfortunately, the ASCA model for the
280 combined dataset showed that no significant spectral variation could be modeled over time to
281 distinguish the three levels of the factor 'infection' (Table 2). On the contrary, the factors 'time' and
282 'plant specimen' resulted significantly different.

283 These results urged us to consider separately the four sampling sessions and to determine the
284 discrimination ability of RS to detect molecular changes induced in leaves by virus infection, on a
285 temporal basis. For this, in order to obtain data grouping in accordance with the infection, at each
286 sampling time, a PCA was performed, i.e. a common visualization method used to reduce the
287 number of variables and to plot multivariate data as a scatter plot accounting for unsupervised
288 agglomeration of samples due to common features. The PCA score plots obtained are shown in
289 Figure 4, colored according to the infection condition at each sampling time.

290 In order to elucidate the spectral features driving this unsupervised clustering of spectra, the
291 loadings of the different PCA models were compared. In details, at T3 and T4, the loadings of the
292 first three PCs are very similar (Figure S2). Noteworthy, the most important features allowing to
293 separate the different spectra are PC1, which refers to the overall spectral intensity, mainly
294 regarding carotenoid peaks, and PC2, accounting to the band shifts observed at 1527 cm^{-1}
295 (carotenoids) and 780 cm^{-1} (aromatics, probably mainly phenolics, such as anthocyanin). This
296 analysis confirms that the differences found in the mean spectra are common to all spectra of the
297 same group, albeit with different magnitude. Moreover, this procedure showed that at T3 and T4 it
298 is possible to distinguish healthy plants from those infected by the two different viruses. On the
299 contrary, at T1 and T2, no score grouping could be obtained in the PC1, 2, 3 scores plot, indicating
300 a poor differentiation of spectral profiles of healthy and infected plants. The variance captured at T1
301 and T2 by the first three PCs, which is mainly related to the fingerprint region between 500 cm^{-1}
302 and 1600 cm^{-1} does not drive clear grouping of scores related to the infection conditions of the
303 samples (Figure S2A and B).

304 **Supervised Data Analysis**

305 Considering the absence of visible symptoms induced on grapevine by the two viruses here
306 considered, a major goal of this work was to determine if RS coupled to multivariate statistical
307 methods could discriminate healthy plants from infected individuals. Therefore, PLS-DA was used
308 as a classification method to evaluate the possibility to discriminate healthy from infected plants
309 based on their Raman spectra. Due to the reduced number of plants included in the experimental
310 design which could not be separated into a calibration and a validation set, the Leave-one-group-out
311 cross-validation (CV) method was used; noteworthy, to test the validity of the model with a method
312 more similar to external set testing, full leave-one-out CV was avoided, and the exclusion groups of
313 CV corresponding to “one-plant-out” at a time were set. Therefore, to test the recognition ability of
314 RS, different class vectors were considered, as follows: (1) three class models (healthy, GRSPaV,
315 GFLV) to simultaneously distinguish healthy plants from plants infected by each of the viruses, (2)
316 two class models (healthy vs. infected plants), considering all infected plants together, and (3) two
317 class models (healthy vs. GRSPaV-infected plants or healthy vs. GFLV-infected plants), separately
318 considering the two different viruses. The classification results of such a cross-validation test are
319 reported in Table 3.

320 Although in the first two measurements (T1 and T2) it was not possible to discriminate the presence
321 of either GRSPaV or GFLV in the plants with a high level of accuracy in CV, infected plants could
322 be distinguished with a classification error (CE) < 20 % starting from the T3 measurement. In
323 particular, infected plants (considering GRSPaV and GFLV together) could be distinguished from
324 healthy individuals with CE values of 8% at T3 and T4, a result particularly relevant considering
325 the complete absence of symptoms. Noteworthy, CE 0% were obtained for GFLV-infected tissue in
326 the last two sampling times, probably resulting from changes in the metabolism of carotenoids
327 occurring in such plants, justifying further investigations, as below described.

328 The score plots of the two best models in the area defined by the two first latent variables (LVs) of
329 the PLS-DA model and the Receiver Operating Characteristic (ROC) curves are shown in Figure 5,
330 providing a clear visual indication of the model sensitivity and specificity. The two relevant LVs of
331 these models are shown in Figure S3, while the model images for the three classes (H,R,G) and for
332 (H,R) at T3 and T4 are reported in Figure S4.

333 Interestingly, the discriminative ability of RS was independent from the amount of virus determined
334 in the leaves and was higher towards the end of the vegetative season (Table 3, Figure 1). This is
335 particularly interesting in the case of GFLV for which the best classification rates in the PLS-DA
336 model were calculated at the T3-T4 measurements against the backdrop of a sharp viral load
337 reduction in the same period. Nonetheless, this result can be assessed in the light of a “load
338 metabolic effect” induced by virus infection in this crop along the seasonal progression (Gambino et
339 al., 2012; Chitarra et al., 2018; Martin et al., 2021). Moreover, the results here reported support
340 previous observations of a higher metabolic impact on grapevine plants exerted by GFLV compared
341 to GRSPaV, corroborating the concept of a co-evolution of GRSPaV with this crop (Gambino et al.,
342 2012) possibly resulting from the long lasting presence of a hard to eradicate pathogen in grapevine.

343 **Validations via Chemical Analytical Extractions**

344 To confirm the results of the RS analyses, the concentration of the three main classes of pigments,
345 i.e. carotenoids, total phenolics, and chlorophylls, were measured by spectrophotometric assays in
346 the same tissues used for RS. As it can be observed in Figure 6, the peculiar trends measured with
347 Raman spectroscopy concerning the concentration of carotenoids were confirmed. In particular, a

348 decrease in carotenoid concentration can be noticed from T1 to T4 in GFLV-infected plants (Figure
349 6A), in accordance with the RS results (Figure 3). Regarding the other two classes of compounds
350 investigated, i.e. chlorophylls and polyphenols, no significant trends are revealed, in line with the
351 observation that their Raman signals were not relevant for the discrimination between healthy and
352 infected plants. However, interestingly, significant differences in the content of total phenolics
353 compounds between healthy and GFLV-infected plants were recorded at T3 and T4, probably
354 supporting the higher discrimination accuracy for infected plants.

355 Regarding chlorophylls, a similar trend was detected over time in all groups of plants,
356 independently on the presence of virus infection. Based on these results, the accumulation of
357 chlorophylls does not seem to be influenced by the infection process, rather by the environmental
358 conditions, while the content of carotenoid and phenolic compounds is altered in infected plants.
359 This observation is in line with recent studies highlighting the relevance of secondary metabolites as
360 players in plant defense responses, thus underlying the importance of characterizing the metabolic
361 profiles associated to disease susceptibility traits in grapevine as a promising approach to identify
362 trait-related biomarkers (Maia et al., 2020).

363 **Transcriptional analysis of genes involved in the carotenoid pathway**

364 Since the most interesting information related to virus infection determined by RS is linked to the
365 carotenoid content, a transcriptional study was conducted by RT-qPCR to measure the expression
366 level of a set of target genes involved in carotenoid metabolism (Leng et al., 2017). Carotenoids are
367 mainly synthesized from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)
368 produced through the monoterpene biosynthetic pathway (MEP). In particular, we tested the first
369 two genes of the biosynthetic MEP route, 1-deoxy-D-xylulose-5-phosphate synthase (*VvDXS*) and
370 1-deoxy-D-xylulose-5-phosphate reductoisomerase (*VvDXR*), and one of the last genes, 1-hydroxy-
371 2-methyl-2-(E)-butenyl-4-diphosphate reductase (*VvHDR*). For these genes, a slight transcript
372 modulation occurred in both healthy and virus-infected plants. While the sampling time (T) was
373 significant for all the three genes, the effect of virus (V) was significant only in the case of *VvDXR*,
374 whose expression increased at T4 in GRSPaV- and GFLV-infected plants. The interaction between
375 virus and time ($V \times T$) was significant only for *VvDXR*, showing a decrease in GFLV-infected
376 plants at T2, followed by an increase at T4 in both virus-infected samples (Figure 7).

377 Two isoforms of geranyl pyrophosphate synthase (*VvGPPS*), a gene operating along the MEP
378 pathway, responsible for the production of geranyl pyrophosphate acting as substrate of
379 monoterpenes synthases in the late carotenoid pathway, resulted strongly transcriptionally regulated
380 along with time progression (T), but not by the presence of virus infection (V). In addition,
381 considering the $V \times T$ interaction, a significant downregulation of *VvGPPS2* was recorded in
382 particular in GFLV-infected plants at T2 (Figure 7), mirroring the carotenoid reduction observed by
383 Raman analysis (Figure 3).

384 Of the two genes encoding the phytoene synthase (*VvPSY*), considered a bottleneck reaction in the
385 carotenoid pathway, *VvPSY1* did not show any significant modulation regarding the effects of virus
386 infection or time progression, while *VvPSY2* showed a strong T effect (Figure 8), indicating its
387 prominent role in the carotenoid reduction occurring after the T1 sampling in the whole set of
388 samples (Figure 3). The phytoene produced by *VvPSY* is then desaturated through the action of
389 phytoene desaturase (*VvPDS*) which showed a modulation affected only by T, in particular at T4.

390 Among the genes involved in carotenoid catabolism, we analyzed a carotenoid cleavage
391 dioxygenase (*VvCCD4*) and a 9-cis-epoxycarotenoid dioxygenase (*VvNCED*). *VvCCD4* is linked to

392 the production of volatile compounds and strigolactones and showed significant V and T effects,
393 with a negative correlation with the accumulation of carotenoids at T3 and T4. On the other side,
394 *VvNCED*, a key enzyme in the biosynthesis of abscisic acid (ABA), showed a significant T effect
395 with negative correlations with the carotenoids at T2 and T3, and an interesting up-regulation in
396 GFLV-infected plants at T4 (Figure 8).

397 Collectively, positive correlations between the reduced accumulation of carotenoids, particularly in
398 GFLV-infected plants, and the down-regulation of transcripts involved in their biosynthesis (i.e.
399 *VvGGPS2* and *VvPSY2*) were detected, accompanied by an up-regulation of genes responsible for
400 carotenoid catabolism, i.e. *VvCCD4* and *VvNCED*. This suggests that virus infection, particularly in
401 the case of GFLV, can accelerate the natural reduction of photosynthetic processes mediated by
402 carotenoids occurring across the vegetative season. Moreover, it indicates that RS can sense a
403 metabolic stress response leading to the accumulation of ABA and strigolactones (Milborrow et al.,
404 1998; Auldridge et al., 2006; Havaux. 2013), originating from carotenoid precursors.

405 CONCLUSIONS

406 A growing number of evidences are showing that RS techniques represent a non-invasive, non-
407 destructive analytical approach to monitor the sanitary status of plants (Payne and Kourowsky,
408 2020). Here, we applied RS to grapevine, one of the most economically important crops worldwide,
409 affected by relatively higher number of pathogens compared to other fruit trees and subjected to
410 strict certification programs to guarantee its phytosanitary status. The PLS-DA model here obtained
411 from the RS data demonstrated the suitability of the RS approach to discriminate healthy from
412 infected plants, even in the absence of macroscopic symptoms, with up to 92% accuracy for
413 GRSPaV and 100% accuracy for GFLV, the latter taken as a representative virus that should be
414 absent in certified virus-free plant materials. The Raman spectra allowed to identify the major
415 metabolic changes occurring in this crop in response to virus infection, paving the way to adopt a
416 RS-based approach as a complementary procedure to detect early stages of viral infection not only
417 in vineyards but also in the nurseries. **Following proper verification of the congruence of the results,**
418 **direct evaluation of plants grown in vineyards will be feasible using high-throughput portable**
419 **Raman spectrometers, as reported by other groups (Farber and Kurouski, 2018; Krimmer et al.,**
420 **2019; Sanchez et al., 2019; Gupta et al. 2020).**

421 DATA AVAILABILITY STATEMENT

422 The raw data supporting the conclusions of this article will be made available by the authors,
423 without undue reservation.

424 AUTHOR CONTRIBUTION

425 LM, CD, SM, FN, GB performed the experiments. LM, EN, GB, GG, AMG, FN analyzed data.
426 LM, GG, and EN wrote the manuscript. LM, GG, AMR, and EN conceived the study and
427 participated in its design. All the authors contributed to the article and approved the submitted
428 version.

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In review

434 **FIGURE LEGENDS**

435 **Figure 1.** Relative accumulation of GRSPaV and GFLV in grapevine cv. Chardonnay leaf tissue, at
436 different times (T1 to T4, at monthly intervals) during the vegetative season. RT-qPCR signals were
437 normalized to *VvAct* and *VvUBI* transcripts. Data are presented as the mean \pm standard error (SE) (n
438 = 4). Lowercase letters denote significant differences attested by Tukey's honestly significant
439 difference (HSD) test ($P < 0.05$).

440 **Figure 2.** Average Raman spectra of healthy (green), GRSPaV- (yellow), and GFLV- (red)-infected
441 grapevine cv. Chardonnay leaves. Spectra are the result of four plants per group. Representative
442 spectra collected in the first measurement session (T1) are shown.

443 **Figure 3.** Raman spectroscopic analysis of healthy and of GRSPaV- or GFLV-infected grapevine cv.
444 Chardonnay leaves, at different time points (T1 to T4) during the vegetative season. Focus on the
445 peaks associated to carotenoids and chlorophyll. Average spectra are the result of four plants per
446 group.

447 **Figure 4.** PCA score plots of the spectra of healthy and GRSPaV- or GFLV-infected grapevine cv.
448 Chardonnay plants, calculated at the different sampling times (T1 to T4, at monthly intervals). 3D
449 graph rotation is set to optimize result visualization.

450 **Figure 5.** PLS-DA model at T3 (A) and T4 (B), with scores on latent variable 1 and 2 plots and
451 Receiver Operating Characteristic (ROC) measurements for GFLV recognition from healthy plants.

452 **Figure 6.** Accumulation of (A) carotenoid, (B) phenolic, and (C) chlorophyll compounds in healthy
453 and infected grapevine leaf samples, during the vegetative season. The values reported are mean \pm
454 standard error (SE) of the classes of compounds obtained from four independent biological samples
455 ($n = 4$).

456 **Figure 7.** Relative expression levels of *VvDXS* (VIT_05s0020g02130), *VvDXR*
457 (VIT_17s0000g08390), *VvHDR* (VIT_03s0063g02030), *VvGGPS1* (VIT_04s0023g01210), and
458 *VvGGPS2* (VIT_18s0001g12000), measured by quantitative reverse transcription-polymerase chain
459 reaction (RT-qPCR). Samples were collected in four sampling points along the season (May_T1,
460 June_T2, July_T3, August_T4). RT-qPCR signals were normalized to *VvAct* and *VvUBI* transcripts.
461 Data are presented as the mean \pm standard error (SE) ($n = 4$). Significance of sampling time, virus,
462 and time x virus ($T \times V$) interaction was assessed by Tukey's HSD test for $P \leq 0.05$ (*), $P \leq 0.01$
463 (**), $P \leq 0.001$ (***) and the corresponding results are given above each graph in the figure panel.
464 Lower case letters above bars are reported when the $T \times V$ interaction are statistically significant as
465 attested by Tukey's HSD.

466 **Figure 8.** Relative expression levels of *VvPSYI* (VIT_04s0079g00680), *VvPSY2*
467 (VIT_12s0028g00960), *VvPDS* (VIT_09s0002g00100), *VvCCD4* (VIT_02s0087g00930), and
468 *VvNCED* (VIT_19s0093g00550), measured by quantitative reverse transcription-polymerase chain
469 reaction (RT-qPCR). Samples were collected in four sampling points along the season (May_T1,
470 June_T2, July_T3, August_T4). RT-qPCR signals were normalized to *VvAct* and *VvUBI* transcripts.
471 Data are presented as the mean \pm standard error (SE) ($n = 4$). Significance of sampling time, virus,
472 and time x virus ($T \times V$) interaction was assessed by Tukey's HSD test for $P \leq 0.05$ (*), $P \leq 0.01$
473 (**), $P \leq 0.001$ (***) and the corresponding results are given above each graph in the figure panel.

474

475 **Table 1.** Raman bands assignments for grapevine leaves.

Band (cm ⁻¹)	Vibrational assignment	References
2800-3000	CH _x stretching	
1605	m v(phenyl ring) (phenolics and lignin)	Eravuchira et al., (2012)
1551	m br chlorophyll - central 16-membered-ring vib.+	
1526	s v1(C-C) (carotenoids)	Koyama et al., (1986)
1483	m δ(CH ₂) and δ (CH ₃)	
1438	m v(phenyl ring) (phenolics)	Eravuchira et al., (2012)
1370	δCH ₂ bending vibration (aliphatic)	Yu et al., (2007).
1328	m δ(CH). v(CN) (pyrrole ring br. - chlorophylls)	Boldt et al., (1987)
1320	[δ(C12 - H), v(C11-C12)](β-carotene)	Eravuchira et al., (2012)
1280	m δ(phenyl-OH) (phenolics) + - δ(CH). v(CN)	Eravuchira et al., (2012); Yeturu et
1215	m δ(CH). δ(CH ₂) (chlorophyll)	Boldt et al., (1987)
1180	ms v(CC). γ(CH) (chlorophylls) + δ(CH phenyl)	Boldt et al., (1987); Eravuchira et
1150	s v2(C\C) (carotenoids)	Gill et al., (1970)
1140	m sh v(CN). δ(CNC) (chlorophyll)	Boldt et al., (1987)
1110	δ (C-OH) (carbohydrates)	Farber and Kurouski, (2018)
1050	v (C-O)+ v(C-C)+ v(C-OH) (carbohydrates)	Farber and Kurouski, (2018)
1000	m δ(C-CH ₃) (carotenoids)	Gill et al., (1970)
980	m undefined (chlorophylls)	
909	m undefined (chlorophylls)	
738	m-s ring br. Mode (aromatics)	

476

477

478 **Table 2.** Results of ASCA elaboration on the complete data set of samples.

Factor	N. of Principals Components	Effect	p-value
Time	3	39.76	0.001
Plant specimen	11	13.57	0.001
Virus	2	2.77	1.00
Mean	-	0.00	-
Residuals	-	52.28	-

479 Results were obtained from 144 spectra collected from 12 plants, over 4 months

480

481 **Table 3.** PLS-DA classification to distinguish grapevine plants infected by either GFLV or
482 GRSPaV from healthy individuals, over the vegetative season.

Model		T1	T2	T3	T4
3 classes	(H,R,G)	50 %	52 %	14 %	19 %
2 classes	(H,I), I=R+G	19 %	36 %	8 %	8 %
2 classes	(H,R)	25 %	31 %	8 %	12 %
2 classes	(H,G)	13 %	17 %	0 %	0 %

483 Results are expressed as classification error (CE) and Cross Validation (CV)

484 H, healthy; R, GRSPaV; G, GFLV; CE, classification error

485

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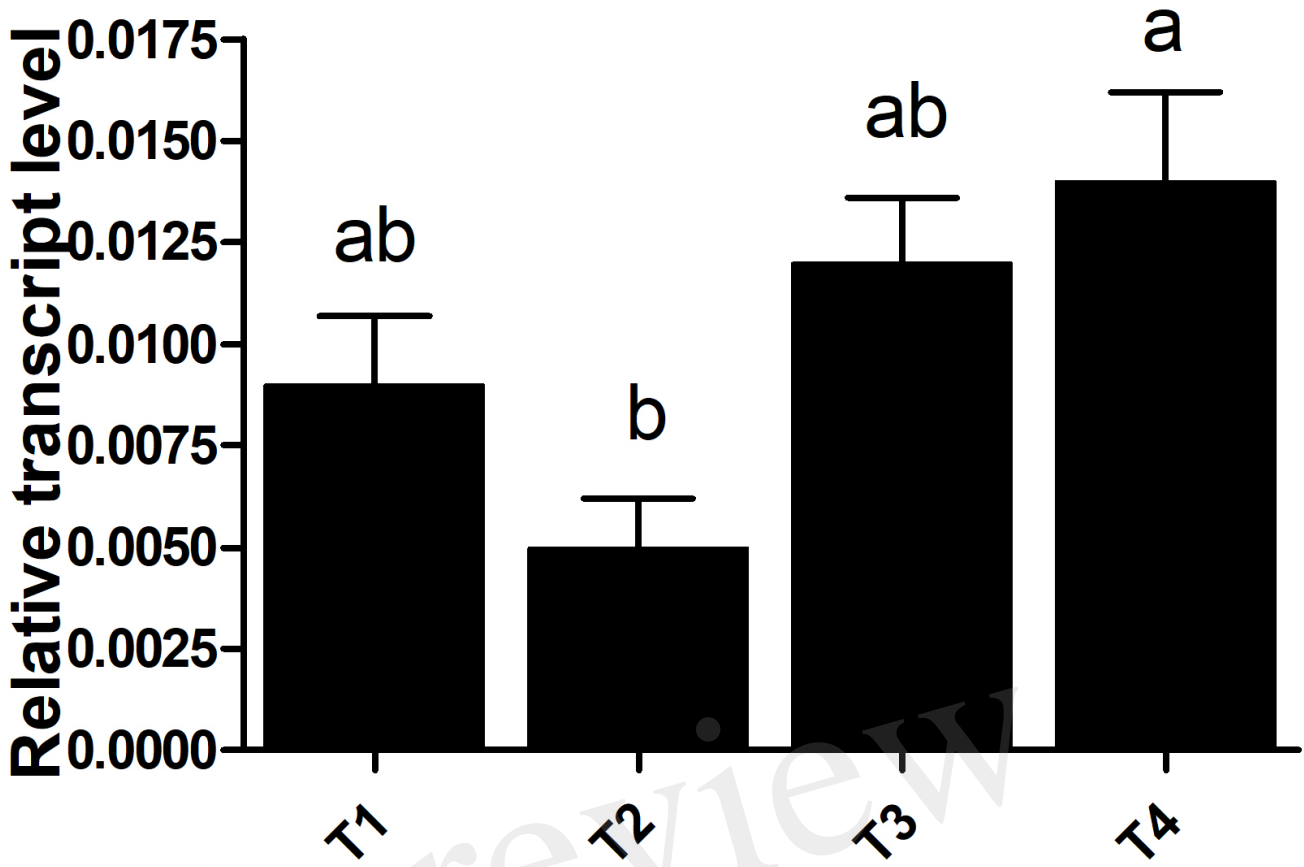
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In review

GRSPaV



GFLV

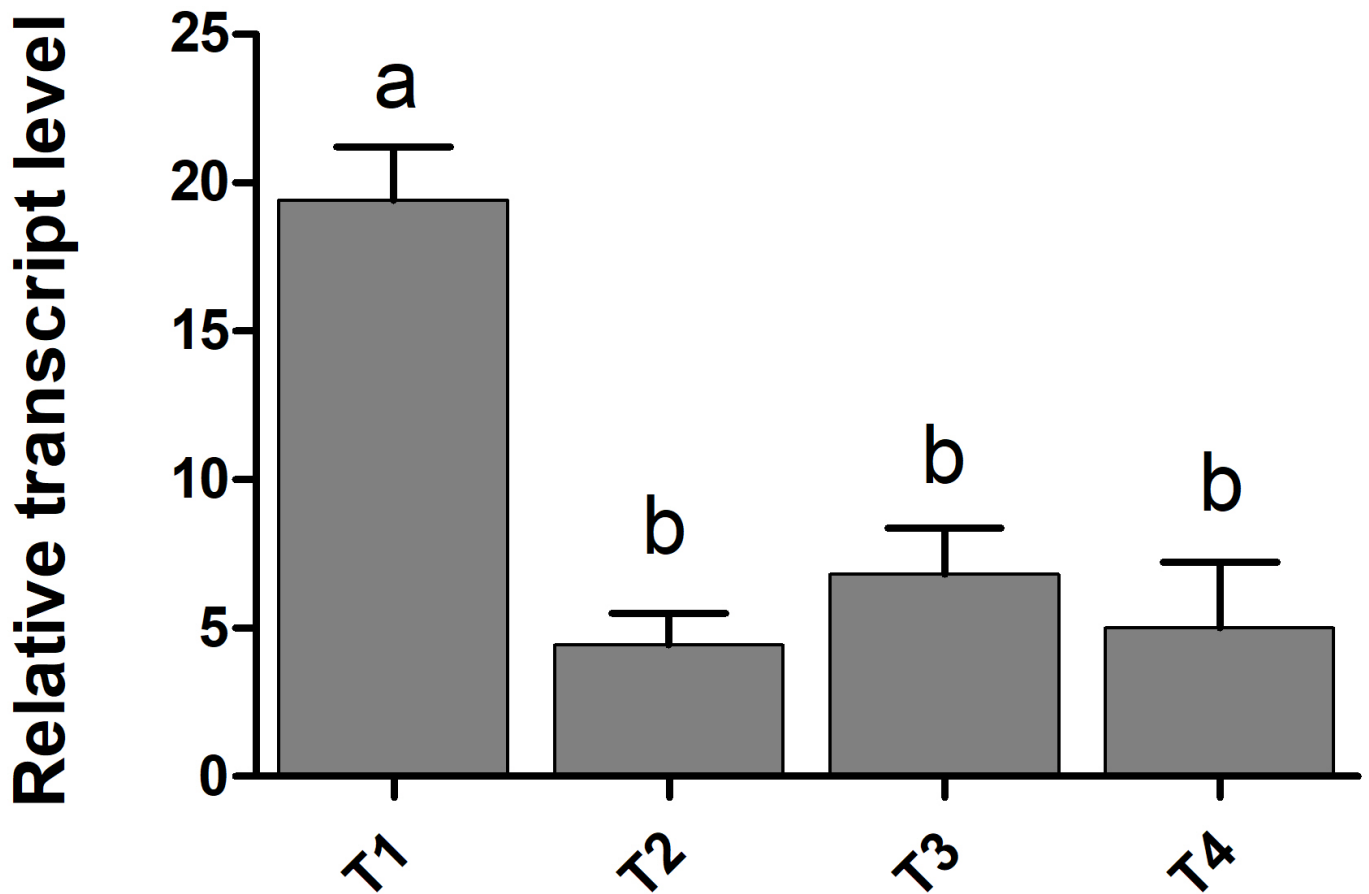


Figure 2.JPEG

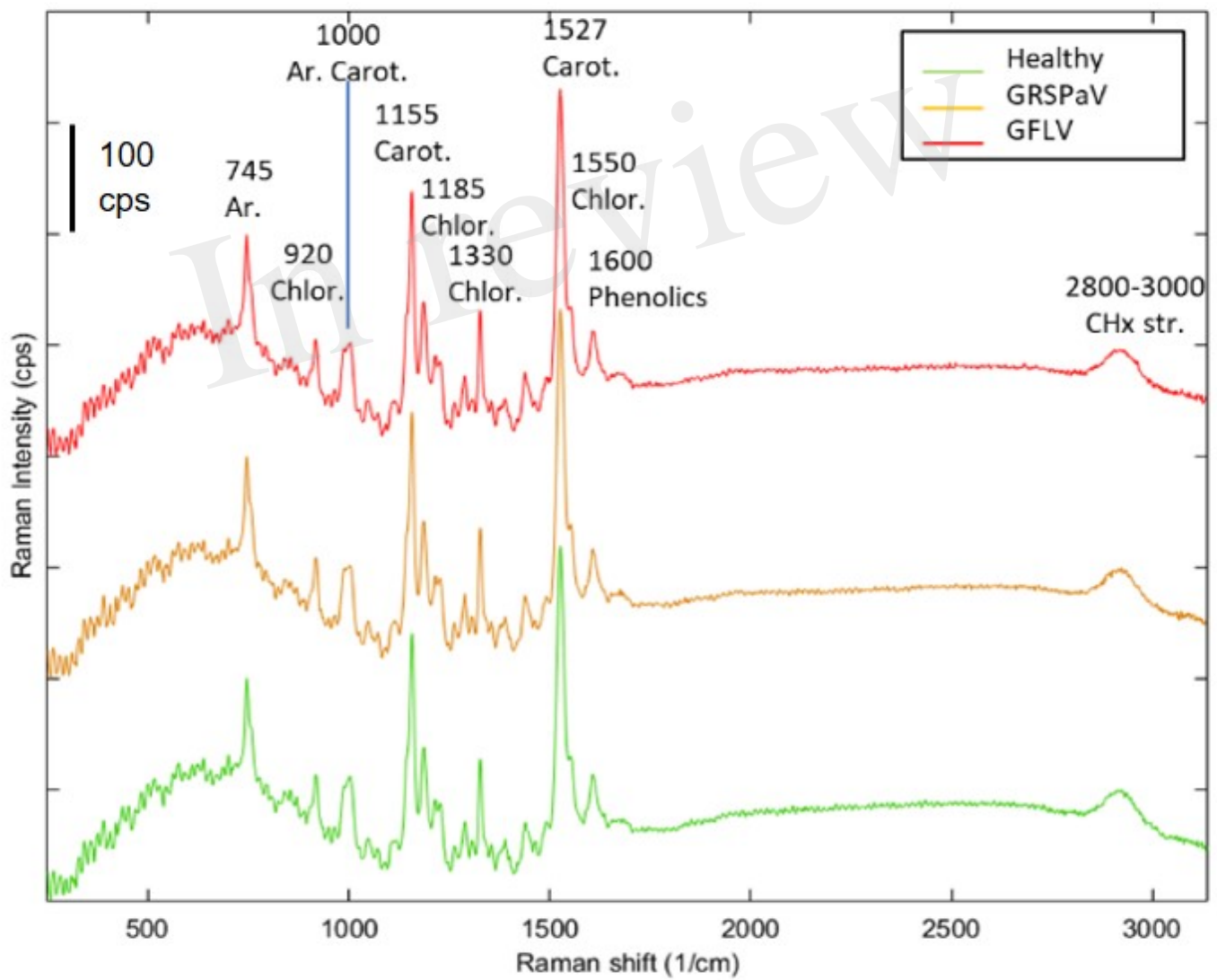


Figure 3.JPEG

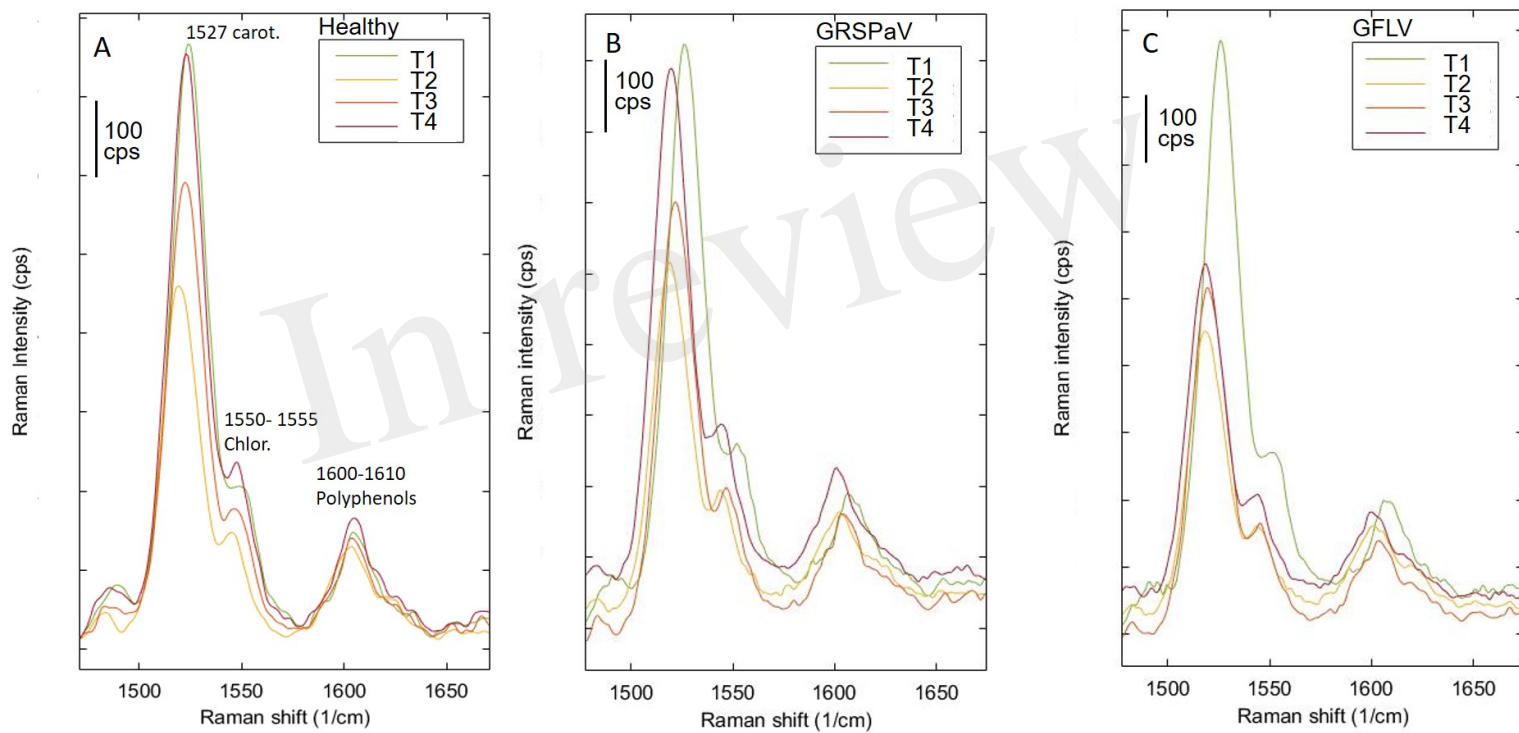


Figure 4.JPEG

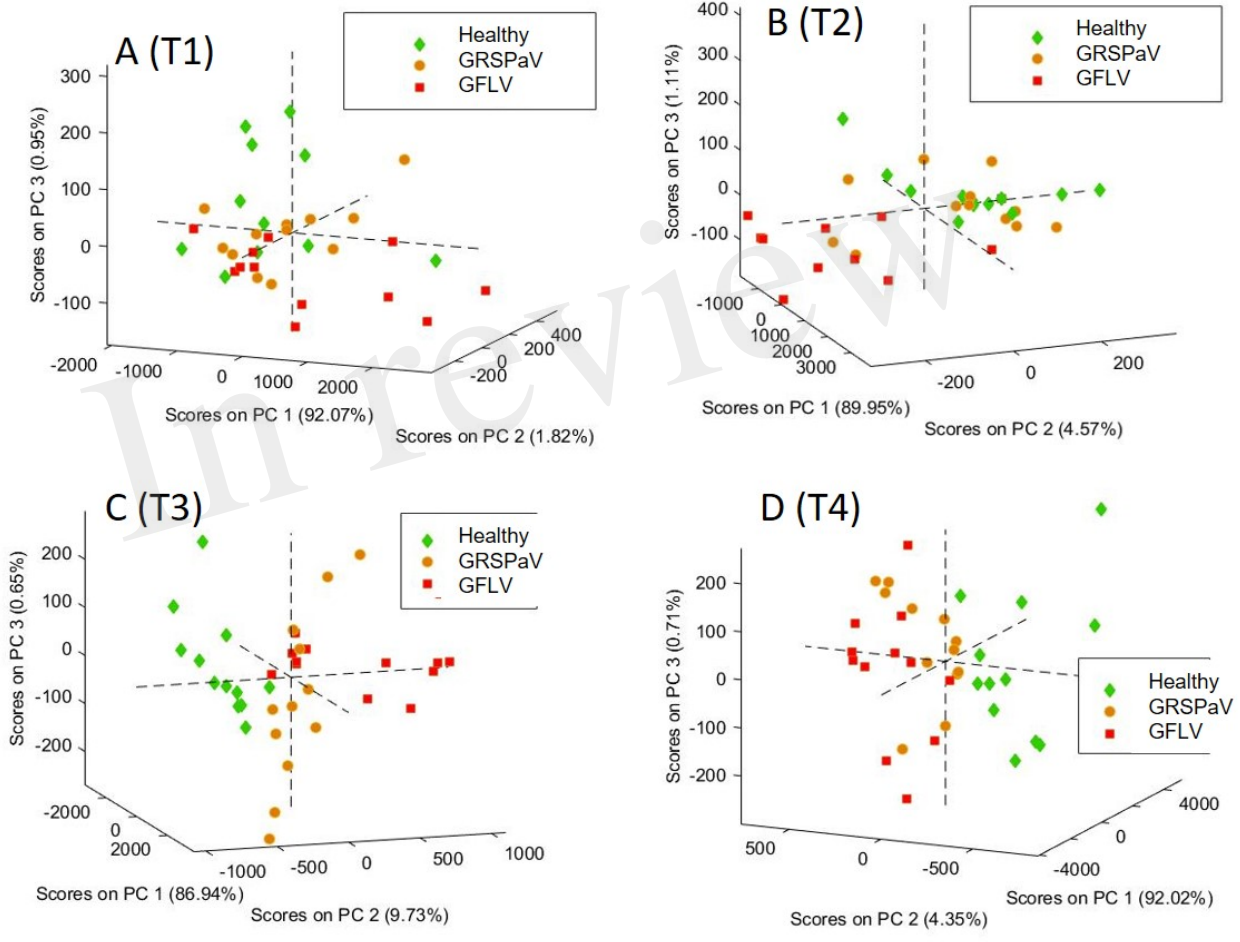


Figure 5.JPEG

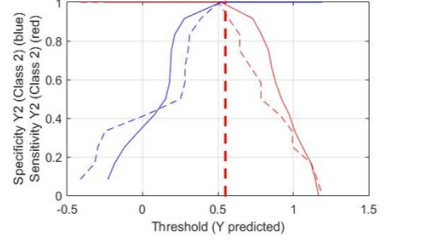
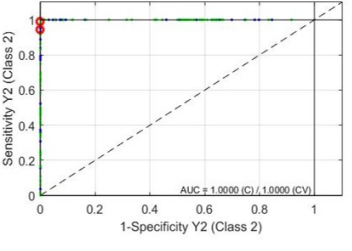
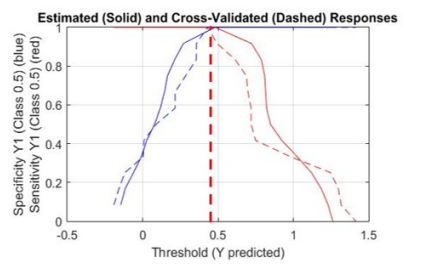
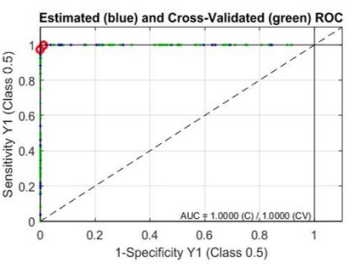
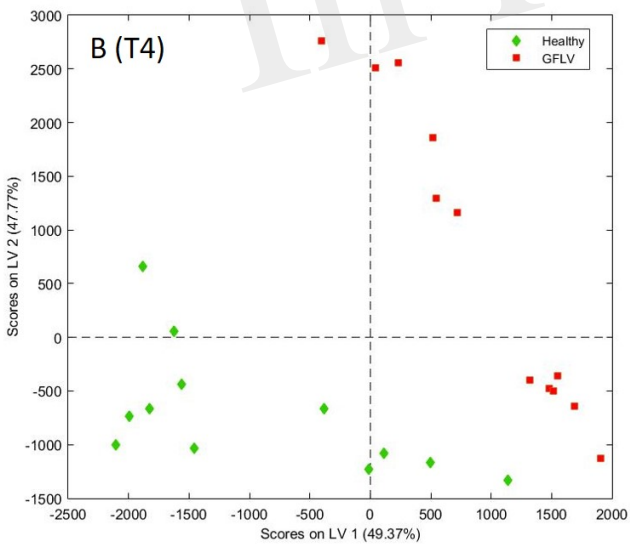
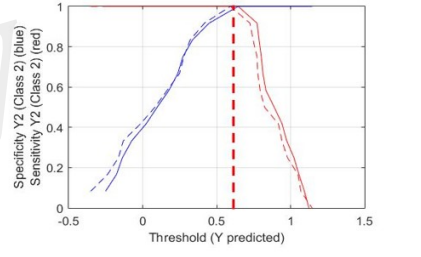
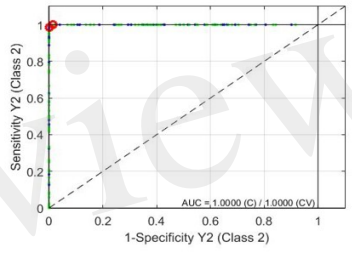
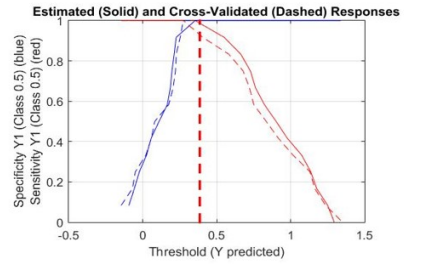
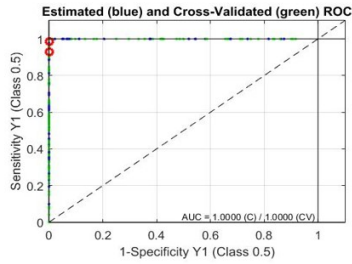
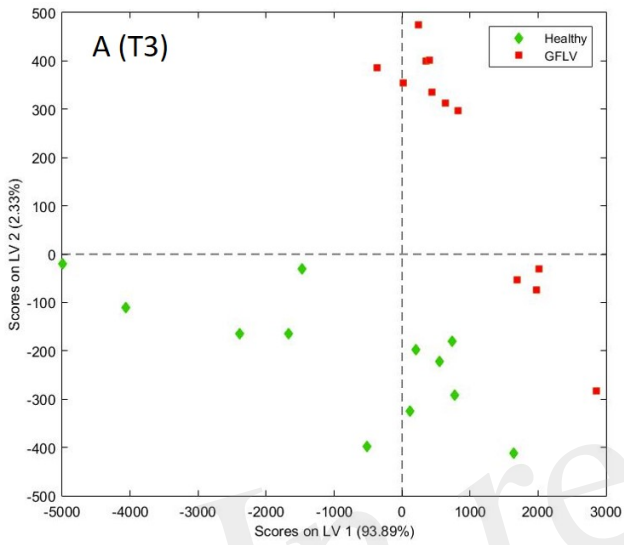


Figure 6.JPEG

In review

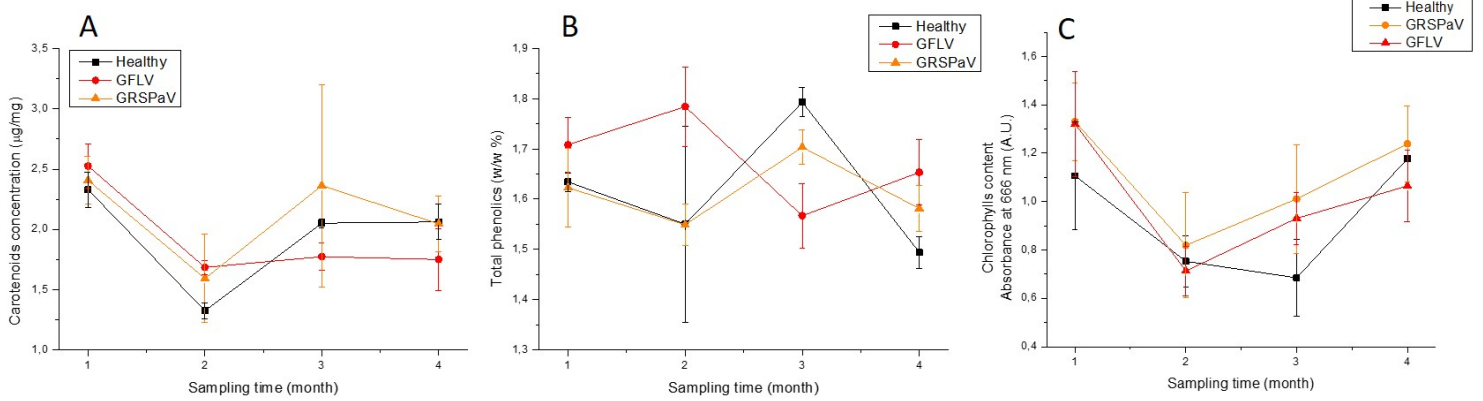


Figure 7.JPEG

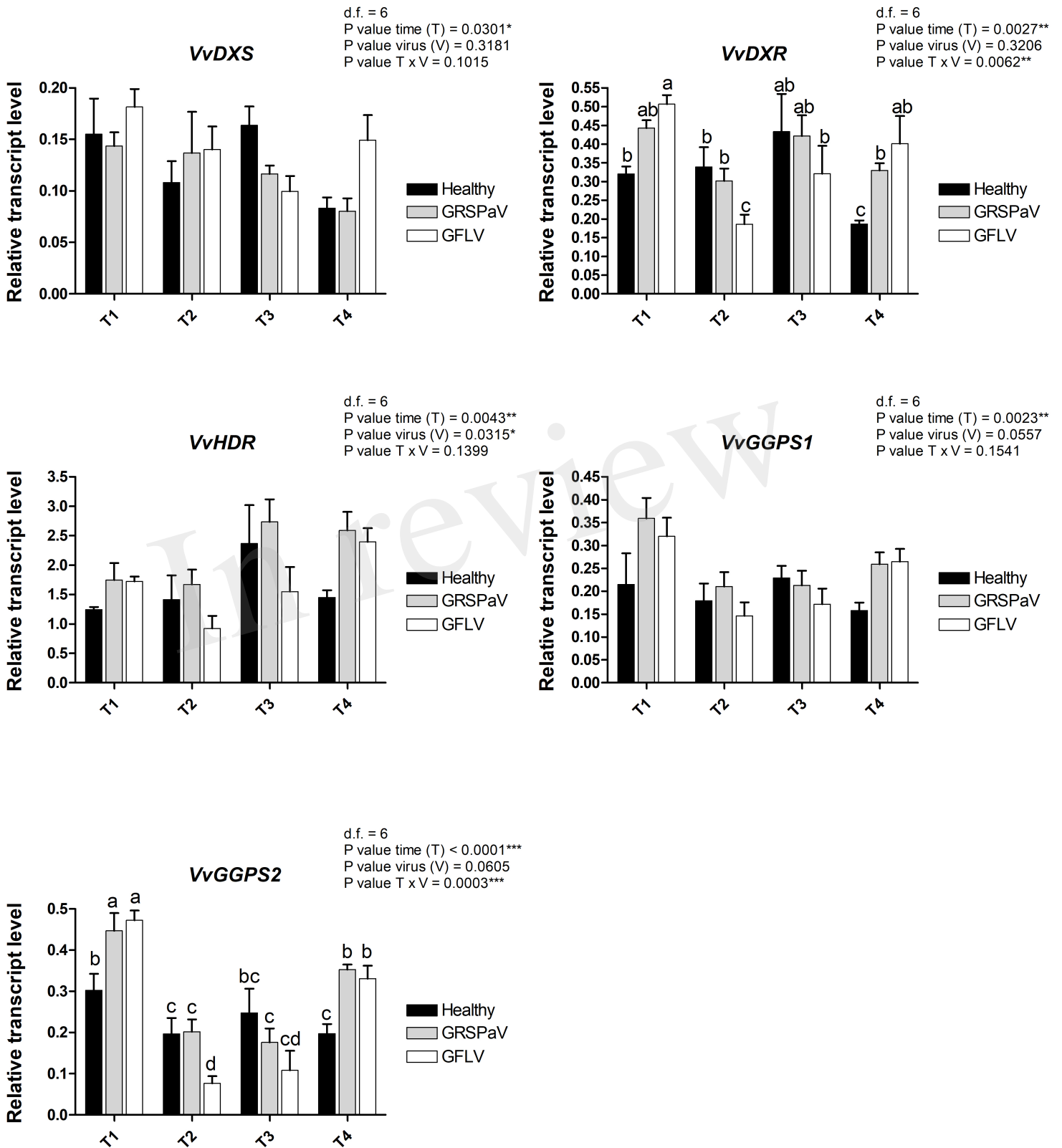


Figure 8.JPEG

