Short title: Grapevine buds' phenolics under chemical stress

Variations in phenolic levels in grapevine buds at eco-dormancy after chemically-induced stress conditions

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¹ Authors' contributions

One sentence summary: Changes in the levels of polyphenol compounds of the grapevine's latent buds hints, for the first time, how much stress the buds actually undergo under chemically-induced stress conditions

¹ I.D., M.S. and K.B. designed the research; I.D. performed the experiments; K.B. supervised the experiments; I.D. and K.S. analyzed the data and performed the statistical analysis; I.D. and M.S. wrote the article with the contribution of K.B.; M.S. agrees to serve as the author responsible and ensures communication. All authors edited and agreed on the final version of the article.

Abstract

Viticulture is one of the briskest economic activities worldwide. A major obstacle impeding such grape yields to satisfy the demand for increased production is the insufficient period of chilling temperatures which, due to climate change, is becoming briefer. The shorter period of cold leads to poor budbreak which, in turn, results in reduced yields. To combat this issue, agronomists have resorted to treatments with chemical regulators to meet the chilling requirement for bud dormancy release several fruit-bearing plants have, including grapevines. This study aimed at identifying and quantifying during eco-dormancy the individual polyphenolic compounds, and their possible variations, in the latent buds of the 'Prime[®]' and 'Ralli' table grape cultivars. The study induced chemical stress by means of four different solutions, at three discrete dates per year, for three consecutive years. Phenolics in the latent buds of the studied varieties were analyzed via HPLC. Their quantitative analysis showed variations both between the varieties and between the samples of those varieties collected after the eight treatments. The analysis indicated that the concentration of phenolics continued progressing during the annual growth cycle of the grapevine, with strong positive correlations being detected between catechin, epicatechin, chlorogenic acid, o-coumaric, piceid, procyanidins B1-B2, rutin, ε-viniferin. Luteolin registered a positive correlation with quercetin, resveratrol, o-coumaric, but not with the remaining polyphenols. The results confirmed that (a) the concentration of phenolics of the latent buds is affected by chemically-induced stress conditions, (b) depending on the date of application, significant changes appear in the variations of those phenolics.

Keywords: eco-dormancy, latent buds, polyphenolic compounds, *Vitis vinifera* L.

76

77 **Introduction**

78

79 Polyphenols, a group of substances with a broad spectrum of physiological activities,
80 are widespread in plants and are also known for their use in traditional medicine and
81 contemporary medical systems. Polyphenolic acids and their derivatives are carbon-
82 based compounds and have been shown to play a role in tissue browning, flavor, and
83 color characteristics of the derived products (Ho et al., 1992). An understanding of
84 polyphenolic composition in fresh fruit; and of the factors that affect polyphenolic
85 compounds are both critical in the design of products and their storage conditions
86 (Oleszek et al., 1994). Consequently, many have been the studies which undertook in
87 the past to investigate the polyphenolic acid compositions of various fruits -and their
88 related cultivars, such as apples and pears (Blankenship et al., 1985; Amiot et al.,
89 1993); *Pyrus* (Challice et. al., 1968); other pome and stone fruits (Herrmann, 1989);
90 *Diospyros* (Ayaz et al., 1997); carrots (Babic et al., 1993); and *Prunus* (Nortje and
91 Koeppen, 1965).

92 Polyphenolic compounds are active biological molecules having one or more
93 benzene rings with one or more hydroxyl functions (Manach et al., 2004). At plant
94 level, polyphenolic compounds contribute to development, cell multiplication,
95 reproduction, differentiation, flowering, and lignification of a plant. As to their
96 concentration in plants, that depends on a number of genetic, physiological and
97 environmental parameters (Balasundram et al., 2007). Polyphenolic compounds
98 accumulate mainly in the cell membrane (lignin and some flavonoids) and in
99 vacuoles where soluble polyphenols such as chlorogenic acid, anthocyanins,
100 flavonols, and tannins are stocked (Peer et al., 2001). At the same time, substances
101 such as polyphenolic compounds, amino acids (namely prolin), and the group of
102 polyamins (PAs) are known to be involved in the resistance response to stress by
103 many plant species, the grapevine included. What is more, they act as free radical
104 scavengers (Bouchereau et al., 1999)

105 The grapevine follows a growth cycle that consists of several steps (Pouget, 1963).
106 The most important of those steps is dormancy, whether it characterizes the
107 dormancy of the latent buds (endo-dormancy) triggered endogenously; or the

dormancy of the vine (eco-dormancy). In the vineyard, eco-dormancy is triggered by a sharp drop in temperature. When cold and low temperatures give rise to challenging environmental conditions for vines during their annual cycle, plant growth slows down, followed by total arrest in autumn and winter. The vines then become dormant, a particular physiological condition that allows them to withstand climate adversities. However, regarding the installation of bud dormancy that takes place earlier during the growth cycle, the decreases in the photoperiod are considered one of the most significant factors among those that can induce that installation (Febvre, 1981; Olsen, 2006; Daskalakis and Biniari, 2019).

The removal of dormancy is also controlled by environmental conditions. When it comes to grapevines, the latent buds acquire the ability to clog under the effect of low temperatures (0 °C – 10 °C). Thus, cold in temperate climates remains the most effective way to break dormancy in woody species (Nigond, 1967; Champagnat, 1983).

Due to the climate change, the current duration of chilling temperatures requisite for bud dormancy release of several fruit plants, grapevines included, suffices no longer. Worse, the brevity of that requisite chilling period appears to be intensifying. As a result, growers and agronomists have been increasingly resorting to treatments of such fruit plants with chemical and growth regulators. Examples include chemicals, used commercially by many wine-producing countries, such as mineral oil, dinitro-O-cresol (DNOC), thiourea, calcium cyanamide, potassium cyanamide, hydrogen cyanamide, and garlic paste (Iwasaki, 1980; Zelleke and Kliever, 1989; Kubota and Miyamuki, 1992; Mizutani et al., 1994; Botelho et al., 2007). Additionally, calcium cyanamide and hydrogen cyanamide have been documented as being quite effective on grape bud dormancy release (Weaver et al., 1961; Iwasaki, 1980; Shulman et al., 1983; Kuroi, 1985; Lin and Wang, 1985). Hydrogen cyanamide is another chemical that has been used successfully to supplement the chilling requirement, significantly improving budbreak (Dokoozlian et al., 1995; Carreno et al., 1999).

To try to comprehend this resting phase of the grapevine, several authors have studied the main biochemical changes the latent buds undergo. In this phenomenon of dormancy, some researchers have made special mention of the involvement of

hydrogen peroxide (H₂O₂) (Lavee and May, 1997), and polyphenolic compounds (Nagar and Sood, 1996; Macheix et al., 2005; Zohra et al., 2011). The evolution of levels in polyphenolic compounds of latent buds during their annual growth cycle seems to be related to that of hydrogen peroxide (H₂O₂). This hypothesis is supported by Li et al. (2011) who reported that the cold treatment leads to higher CAT (catalase) and APX (ascorbate peroxidase) activities in the leaves, since polyphenolic compounds are essentially an anti-stress mechanism. However, so far, no studies have been carried out on the individual polyphenolic compounds in the latent buds of the grapevine during eco-dormancy and, more importantly, under chemically-induced stress conditions. Thus, the purpose of the present study was (a) to examine the premise that oxidative stress is caused by applications with hydrogen cyanamide which advance budbreak; and (b) to monitor, for the first time during such experiments and during the annual vegetative cycle, the variations in the levels of thirteen (13) individual polyphenolic compounds of the latent buds in two (2) grapevine varieties 'Prime[®]' and 'Ralli' (*Vitis vinifera* L.), also in relation to the different time of applications and the two different chemical substances used (Theocopper & Erger), for three consecutive years.

Materials and Methods

Plant material and experimental design

'Prime[®]' is a white table grape variety and 'Ralli' is a red table grape variety (*Vitis vinifera* L.). Both are regarded by viticulturists worldwide as two of the grapevine varieties with the earliest maturation. The present study's researchers located seven-year old vines of these two varieties in a vineyard in Corinth (alt: 10 m, gradient: 2%), northeastern Peloponnese, Greece. The selected vines (already grafted on rootstock 1103 Paulsen), were bilaterally cordon-trained (bilateral Royat) at 2.2 m x 1.2 m intervals, and cane-pruned to 10-node canes per arm. Each vine consisted of four (4) arms and, therefore, each vine comprised four (4) canes in total. The usual viticultural techniques entailed: fertilization using 11-15-15 NPK at a dose

of 250 g/vine; canopy management techniques (shoot thinning, topping; girdling); and irrigation. All studied vines had been grown in the same area and under the same climate and soil conditions.

173

174 *Treatments*

The experiment lasted three (3) consecutive years and, more specifically, during the 2016-2018 cultivation seasons. Four (4) different solutions (Theocopper & Theocal, Dormex, Erger, and garlic extract), known for their ability to advance budbreak, were applied to and evaluated for two table grape varieties, 'Prime[®]' and 'Ralli'. Eight (8) preparations were applied to each variety at three different dates (December 15, January 15, and February 15), in three (3) consecutive years, bringing the total of treatments to one hundred and forty-four (144). In other words, the two (2) varieties were treated to eight (8) applications, on three different (3) dates, for three (3) consecutive years ($2 \times 8 \times 3 \times 3 = 144$). The groups of ten (10) vine canes selected were chosen not only because of their morphology but also because they were the most representative of each variety. For the needs of the experiment, the research made use of a Randomized Complete Block Design with three (3) replications per treatment. Each group of 10 vine canes constituted one (1) replication.

The eight applications were effected using the following mixtures:

- 189 (i) 10 mL Theocopper and 1 g Theocal per Liter [TA];
- 190 (ii) 20 mL Theocopper and 1 g Theocal per Liter [TB];
- 191 (iii) 25 mL Dormex[®] per Liter [DA];
- 192 (iv) 50 mL Dormex[®] per Liter [DB];
- 193 (v) 35 mL Erger and 80 mL Active Erger per Liter [EA];
- 194 (vi) 70 mL Erger and 160 mL Active Erger per Liter [EB];
- 195 (vii) extract from 300 g (FW-fresh weight) garlic evaporated and then diluted in 1 L
- 196 water [GA]; and
- 197 (viii) distilled water (control treatment) [Control].

198

Preparations were applied using a knapsack sprayer. The synthesis of the preparations was as follows: Theocopper: 10% sugars, 10% amino acids, 12% urea,

201 1.4% potassium in organic form, 12% nitrogen in organic form, and 3.5% organic
202 matter; Theocal: 30% calcium, 35% organic matter with a pH of 7.1. Dormex®: H₂CN₂
203 490 g L⁻¹ (BASF Co.)

204 As to the garlic extract, that was prepared as follows: 300 g of fresh and peeled
205 cloves of garlic (*Allium sativum* L.) were ground in a blender. The ensuing mash was
206 blended again with 1 L distilled water, filtered, and raised to 2 L by the addition of
207 distilled water to obtain a final concentration of 15% garlic extract (Kubota and
208 Miyamuki, 1992).

209

210 *Sampling*

211 The canes were sampled on day 0, i.e., before the first application, and then on day
212 3, 6, 9, 15, and 30 following each application. As stated earlier, the applications took
213 place on three different dates (December 15; January 15; February 15) with the
214 sprayings focusing on the first ten (10) latent buds starting at the base of the canes.
215 The samples were randomly collected from the entire vineyard so that sampling may
216 be more homogeneous and representative of the space the samples had been grown
217 in. Once collected, they were frozen in liquid nitrogen and then lyophilized. Last,
218 buds were homogenized in liquid nitrogen using a pre-chilled mortar and pestle for
219 the extraction.

220

221 *Reagents and chemicals*

222 The various polyphenolic compounds analyzed were identified according to their
223 order of elution and the retention times of the pure compounds. Non-colored
224 polyphenolics were purchased from a number of different sources. More specifically,
225 catechin, vanillic acid, chlorogenic acid, epicatechin, o-coumaric acid, and rutin were
226 procured from Sigma, St. Louis, MO, USA. Luteolin, procyanidin B1, procyanidin B2,
227 ε-viniferin, quercetin, trans-resveratrol, and piceid were purchased from
228 Extrasynthese, Gemay, France.

229

230 *Extraction of polyphenols*

231 The procedure followed in order to extract the polyphenols from each and every
232 sample was the following: 5 mL of 70% v/v methanol acidified with 1% formic acid
233 (v/v) were added to 200 mg of dried tissue which were then weighed on a precision
234 scale (KERN 410). The mixture was shaken and placed in a water bath for 60 min at a
235 temperature of 40 °C and centrifuged at 4,000 rpm for 6 min. Once the supernatants
236 (5 mL) were collected, the same extraction process was repeated twice more for the
237 remaining polyphenols, resulting in a total extraction volume of 15 mL 70% v/v
238 methanol. All fractions were combined. Last, the supernatants were stored at a
239 temperature below 80 °C until the time of the analysis.

240

241 *Analysis by HPLC*

242 In order to measure individual polyphenols with HPLC, liquid extraction was carried
243 out: 2 mL of the 70% v/v methanol extract were evaporated with a sample
244 concentrator at room temperature under a stream of nitrogen gas. By means of that
245 method, the tubes held nothing but the aqueous residue containing the polyphenolic
246 compounds. Next, 1 mL water (HPLC grade) was added to that residue, followed by
247 vortex. Then, 2 mL of ethyl acetate were added to the mixture followed by vortex for
248 1 min. The following step entailed transferring the supernatant ethyl acetate to a
249 new tube, adding 2 mL of ethyl acetate to the pellet, followed by vortex for 1 min.
250 The new supernatant ethyl acetate was transferred and mixed with the previous
251 one. The combined ethyl acetate supernatant was evaporated with a sample
252 concentrator at room temperature under a stream of nitrogen gas and the pellet
253 was dissolved in 1 mL of methanol (HPLC grade). Last, prior to the HPLC analysis, the
254 extract was filtered through a 0.22 µm membrane.

255 Monomeric and dimeric phenols [(+)-catechin, (-)-epicatechin, procyanidins B1 and
256 B2, vanillic acid, o-coumaric, chlorogenic acid, piceid, rutin, trans-resveratrol, ε-
257 viniferin, quercetin, and luteolin] were determined by the HPLC-DAD system
258 (Shimadzu Nexera).

259 For the separation of monomeric and dimeric polyphenols, the study employed a
260 250 x 4.6 mm ID, 5 µm, Waters x select C18 column operating at 25°C. The eluent
261 comprised (a) H₂O/ C₂H₄O₂ (99.3:0.7); and (b) CH₃OH (100). The flow rate stood at

262 0.5 mL min⁻¹. The linear gradient program used for the elution was the one described
263 in Biniari et al. (2020).

264

265 *Data analysis*

266 The experiment was designed and implemented following the principles of the
267 Randomized Complete Block Design. Data were analyzed using the Statgraphics
268 Centurion statistical package (Statgraphics Technologies, Inc., version 17) and are
269 presented as mean ± SE (Standard Error) of the three (3) replications. Each group of
270 ten (10) grapevine canes constituted one (1) replication.

271 A repeated measures General Linear Model (GLM) was applied to the data
272 (polyphenolic compounds) considering the sampling time as repeated measure, with
273 fixed effects of the treatment (control, TA, TB, DA, DB, EA, EB, GA), the grapevine
274 variety (Prime[®], Ralli), the year of the experiment (2016, 2017, 2018), the dates
275 when the applications were performed (December 15, January 15, and February 15)
276 and number of days that had elapsed between applications and sampling (0, 3, 6, 9,
277 15, 30 days). *Post hoc* analyses were performed using Tukey's HSD test. The
278 Kolmogorov-Smirnov test revealed that all variables followed normal distribution.
279 Moreover, in order to reduce the dimensionality of the data and investigate the
280 relationships between and among polyphenolic compounds, pooled data (all
281 polyphenols) were subjected to principal component analysis (PCA). Results and
282 their detailed description are shown in the present study's tables and diagram (plot).
283 The significance level for all tests was set at 5%

284

285 **Results and Discussion**

286

287 According to their respective retention time, thirteen (13) polyphenolic compounds
288 were identified: (1) procyanidin B1; (2) procyanidin B2; (3) catechin; (4) chlorogenic
289 acid; (5) vanillic acid; (6) epicatechin; (7) piceid; (8) rutin; (9) o-coumaric; (10)
290 resveratrol; (11) ε-viniferin; (12) quercetin; and (13) luteolin. The polyphenolic
291 content is expressed as µg equivalent per g of dry weight (µg g⁻¹ dw).

The content of polyphenols in 'Prime[®]' and 'Ralli', in tandem with (i) the year of the experiment; and (ii) the month when the applications were performed, is presented in Table 1. The content of all individual polyphenolic compounds in the two varieties under study registered their highest point in the application effected in February, with the exception of vanillic acid and epicatechin whose content peaked in January (Table 1). With regard to the year when the experiment took place, the results showed that polyphenolics procyanidin B1, catechin, vanillic acid, epicatechin, piceid, rutin, and ϵ -viniferin exhibited their highest concentration in 2016, the first year of the experiment (Table 1).

301

Table 1: Variation of individual polyphenolic compounds in the buds at eco-dormancy in relation to the year and month the applications were carried out

304

	Year					Month				
	2016	2017	2018	SEM	P-Value	December	January	February	SEM	P-Value
Procyanidin B1	1284.5 ^c	850.9 ^a	970.8 ^b	7.21	<0.001	955.3 ^a	1032.1 ^b	1118.6 ^c	7.15	<0.001
Procyanidin B2	877.1 ^a	966.6 ^b	961.9 ^b	8.11	<0.001	875.7 ^a	898.8 ^a	1031.1 ^b	8.04	<0.001
Catechin	816.7 ^c	541.6 ^b	384.8 ^a	6.78	<0.001	513.9 ^a	612.1 ^b	617.1 ^b	6.72	<0.001
Chlorogenic acid	214.3 ^{ab}	219.1 ^b	211.1 ^a	1.87	<0.001	188.8 ^a	203.8 ^b	251.7 ^c	1.85	<0.001
Vanillic acid	126.1 ^c	65.66 ^a	77.88 ^b	1.19	<0.001	77.31 ^a	97.69 ^b	94.65 ^b	1.17	<0.001
Epicatechin	2216.9 ^c	1124.6 ^a	1724.0 ^b	9.85	<0.001	1488.6 ^a	1843.8 ^b	1733.1 ^c	9.77	<0.001
Piceid	125.3 ^c	99.45 ^b	74.58 ^a	0.88	<0.001	94.24 ^a	97.02 ^b	108.1 ^c	0.88	<0.001
Rutin	2206.5 ^c	1866.0 ^b	1606.7 ^a	26.92	<0.001	1788.2 ^a	1809.5 ^a	2081.4 ^b	26.7	<0.001
o-coumaric	160.1 ^b	134.1 ^a	178.5 ^c	2.16	<0.001	130.9 ^a	155.4 ^b	186.4 ^c	2.14	<0.001
Resveratrol	145.2 ^a	161.1 ^b	161.2 ^b	1.77	<0.001	121.2 ^a	162.7 ^b	183.6 ^c	1.75	<0.001
ϵ-viniferin	504.1 ^c	280.9 ^b	213.4 ^a	2.74	<0.001	304.3 ^a	325.2 ^b	369.6 ^c	2.72	<0.001
Quercetin	181.4 ^a	232.3 ^b	301.1 ^c	2.58	<0.001	229.6 ^a	234.4 ^a	250.7 ^b	2.56	<0.001
Luteolin	88.64 ^a	112.2 ^b	192.4 ^c	1.36	<0.001	115.6 ^a	134.1 ^b	143.5 ^c	1.34	<0.001

305

Values are the means of triplicates. Values on the same line carrying a different superscript (a - c) are significantly different at $P < 0.05$. The results are expressed as μg equivalent per g of dry weight ($\mu\text{g g}^{-1}$ dw)

309

The polyphenolic content in 'Prime[®]' and 'Ralli' as it relates to (i) the studied variety; and (ii) the lapse of days between applications and cane sampling, is presented in

Table 2.

When compared to 'Ralli', 'Prime[®]' is characterized by a greater concentration in almost all polyphenolics, with the exception of resveratrol and luteolin. Despite the fact that both 'Prime[®]' and 'Ralli' are early maturation varieties, 'Prime[®]' sprouts even earlier than 'Ralli'. Thus, it can be surmised that 'Prime[®]' is more sensitive to stress than 'Ralli' (Table 2).

The distribution (%) of analyzed polyphenolic compounds within the studied varieties in relation to the lapse of days between applications and cane sampling is presented in Table 2. The results showed that the highest concentrations were observed in the samplings that took place on the 15-day and 30-day mark following the applications.

The total amounts of polyphenolic compounds increase in early July and, by the end of that phase (pre-dormancy), those amounts start decreasing, coinciding with the start of the endo-dormancy phase (Said et al., 2014). During that phase, the amounts of polyphenolic compounds gradually decrease, while the amount of polyphenolic compounds in latent buds dramatically decreases. That rapid decrease occurs within a short period of time that coincides with the end of the endo-dormancy phase when temperatures remain below 10°C for at least seven (7) successive days. Afterwards, their total amounts start increasing slightly until budburst. That moderate increase corresponds to the buds' dehydration phase (Said et al., 2014).

Table 2: Variation of individual polyphenolic content in relation to the variety under study and the lapse of days between applications and cane sampling

	Variety				Sampling after							
	'Prime [®] '	'Ralli'	SEM	P-Value	0 day	3 days	6 days	9 days	15 days	30 days	SEM	P-Value
Procyanidin B1	1088.8 ^b	981.8 ^a	5.84	<0.001	677.1 ^a	1064.2 ^b	1075.3 ^b	1142.9 ^c	1118.4 ^c	1134.2 ^c	9.91	<0.001
Procyanidin B2	1019.8 ^b	850.6 ^a	6.56	<0.001	637.8 ^a	982.9 ^b	966.6 ^b	1030.7 ^c	987.9 ^{bc}	1005.1 ^{bc}	11.14	<0.001
Catechin	634.8 ^b	527.3 ^a	5.49	<0.001	378.7 ^a	600.5 ^c	561.1 ^b	666.1 ^d	605.1 ^c	674.7 ^d	9.32	<0.001
Chlorogenic acid	249.4 ^b	180.1 ^a	1.51	<0.001	148.7 ^a	214.2 ^b	219.1 ^{bc}	230.6 ^d	228.9 ^{cd}	247.2 ^e	2.57	<0.001
Vanillic acid	101.1 ^b	78.62 ^a	0.96	<0.001	57.33 ^a	94.73 ^b	93.01 ^b	96.99 ^b	98.5 ^b	98.76 ^b	1.63	<0.001
Epicatechin	1836.1 ^b	1541.1 ^a	7.98	<0.001	1249.9 ^a	1706.9 ^b	1754.7 ^{bc}	1768.4 ^c	1739.8 ^{bc}	1911.3 ^d	13.55	<0.001
Piceid	109.1 ^b	90.54 ^a	0.72	<0.001	65.91 ^a	100.6 ^b	109.2 ^c	107.9 ^c	106.2 ^c	108.8 ^c	1.22	<0.001
Rutin	2149.3 ^b	1636.8 ^a	21.81	<0.001	1186.2 ^a	2044.2 ^{bc}	1967.7 ^b	1965.7 ^b	2188.1 ^c	2006.5 ^b	37.02	<0.001
o-coumaric	165.8 ^b	149.3 ^a	1.75	<0.001	84.59 ^a	163.3 ^b	170.6 ^{bc}	174.4 ^{bc}	173.5 ^{bc}	178.9 ^c	2.97	<0.001

Resveratrol	141.3 ^a	170.4 ^b	1.43	<0.001	86.99 ^a	153.1 ^b	163.6 ^c	165.1 ^c	177.9 ^d	188.3 ^d	2.44	<0.001
ε-viniferin	343.5 ^b	322.6 ^a	2.23	<0.001	209.1 ^a	340.4 ^b	350.6 ^{bc}	361.2 ^{cd}	368.6 ^d	368.3 ^d	3.77	<0.001
Quercetin	248.2 ^b	228.3 ^a	2.09	<0.001	160.9 ^a	247.9 ^b	249.1 ^b	261.2 ^b	255.6 ^b	254.5 ^b	3.55	<0.001
Luteolin	119.7 ^a	142.4 ^b	1.09	<0.001	90.29 ^a	137.6 ^{bc}	140.4 ^{cd}	132.9 ^b	139.3 ^{bcd}	145.9 ^d	1.86	<0.001

335 Values are the means ($\mu\text{g g}^{-1}$ dw) of triplicates. Values on the same line carrying a different superscript
336 (a - d) are significantly different at $P < 0.05$. The results are expressed as μg equivalent per g of dry
337 weight ($\mu\text{g g}^{-1}$ dw)

338

339 The polyphenolic content, in relation to the treatments applied to the 'Prime[®]'
340 variety, is presented in Table 3, while that of the 'Ralli' variety, again in relation to
341 the treatments applied, is presented in Table 4.

342

343 **Table 3:** Variation of individual polyphenolic content in relation to the treatment applied on
344 the buds of the 'Prime[®]' variety

	Treatments ('Prime [®])									
	Control	Garlic	DA	DB	EA	EB	TA	TB	SEM	P-Value
Procyanidin B1	810.7 ^a	976.8 ^b	976.7 ^b	1213.3 ^d	1089.3 ^c	1308.5 ^e	1068.5 ^c	1269.9 ^{de}	15.65	<0.001
Procyanidin B2	741.2 ^a	918.7 ^b	825.4 ^a	1130.9 ^c	961.4 ^b	1332.2 ^d	981.6 ^b	1260.9 ^d	19.77	<0.001
Catechin	437.4 ^a	541.9 ^{bc}	506.6 ^b	734.1 ^d	576.1 ^c	827.5 ^e	605.2 ^c	858.2 ^e	15.99	<0.001
Chlorogenic acid	190.2 ^a	221.1 ^b	218.8 ^b	265.1 ^d	236.9 ^{bc}	291.9 ^e	245.7 ^c	331.5 ^f	4.51	<0.001
Vanillic acid	69.28 ^a	81.68 ^b	80.13 ^{ab}	112.6 ^d	87.77 ^{bc}	132.6 ^e	97.95 ^c	146.1 ^f	2.81	<0.001
Epicatechin	1435.6 ^a	1626.4 ^b	1738.7 ^c	2047.4 ^d	1795.4 ^c	2140.1 ^d	1776.3 ^c	2133.2 ^d	22.99	<0.001
Piceid	81.34 ^a	89.29 ^{ab}	93.99 ^{bc}	122.9 ^e	103.9 ^d	151.1 ^e	99.76 ^{cd}	133.9 ^f	2.07	<0.001
Rutin	1503.5 ^a	1812.3 ^a	1788.7 ^a	2333.2 ^b	2160.3 ^b	2711.2 ^c	2212.1 ^b	2768.6 ^c	75.01	<0.001
o-coumaric	114.1 ^a	135.2 ^{bc}	133.3 ^{ab}	197.6 ^e	161.9 ^d	211.5 ^{ef}	154.5 ^{cd}	223.6 ^f	4.57	<0.001
Resveratrol	103.1 ^a	117.5 ^b	116.1 ^b	165.8 ^d	139.5 ^c	197.3 ^f	124.7 ^b	179.7 ^e	2.86	<0.001
ε-viniferin	247.8 ^a	304.2 ^{bc}	297.2 ^b	373.8 ^e	325.6 ^{cd}	440.9 ^f	331.5 ^d	437.6 ^f	6.34	<0.001
Quercetin	184.8 ^a	223.3 ^b	215.7 ^b	276.8 ^c	228.1 ^b	318.2 ^d	236.5 ^b	319.4 ^d	5.23	<0.001
Luteolin	94.6 ^a	100.4 ^{ab}	110.7 ^{bc}	123.6 ^d	121.6 ^{cd}	136.1 ^{ef}	133.1 ^{de}	146.1 ^f	2.87	<0.001

345 Values are the means ($\mu\text{g g}^{-1}$ dw) of triplicates. Values on the same line carrying a different superscript
346 (a - f) are significantly different at $P < 0.05$. The results are expressed as μg equivalent per g of dry
347 weight ($\mu\text{g g}^{-1}$ dw)

348

349 Compared to the control treatment, all other treatments produced an increase in the
350 content of polyphenolic compounds (Tables 3, 4). In all likelihood, the evolution of
351 polyphenolic compounds in the latent buds of the vine during the plant's annual
352 growth cycle appears to be associated with different development phases (Pouget,

1963) and with abiotic stress as applied by the environment. In fact, the accumulation of those compounds during the phase of dormancy could be linked to the decrease in temperature and shorter length of daylight. Such results have also been suggested by many researchers (Macheix et al., 2005; Wahid and Ghazanfar, 2006) who have shown that certain polyphenolics also play a role in the plant's tolerance to abiotic stress and argue that the temperature, as a polyphenolic metabolism expression regulator, induces an accumulation of anthocyanins. As such, this regulation may intervene at the level of activity Phenylalanine (PAL) (Macheix et al., 1990).

The accumulation of polyphenolic compounds seems to be associated with low temperatures, characterizing the phase of eco-dormancy when temperatures stay below 10°C for a period of 10 days (Pouget, 1963). Such a hypothesis seems in agreement with the findings of Pennycooke et al. (2005) who showed that the cold caused an accumulation of polyphenolic compounds in *Betunia hybrida* leaves. Wróbel et al. (2005) have also shown that the cold stimulates some polyphenolic compounds (free gallic acid and catechin) in the seeds of *Vitis riparia*.

According to the authors cited above, once those polyphenolic compounds metabolize, they are transferred to other parts of the plant to meet the plant's developmental requirements. Such results were also yielded by Zohra et al. (2011) who reported that levels of quercetin in the grapevine variety Carignan increase during the phase of dormancy and decrease during the phase of budbreak. Be that as it may, the results of the present experiment did not confirm those findings since, in the case of the present study, quercetin increased during eco-dormancy and spiked once the treatments were effected (Table 4).

377

Table 4: Variation of individual polyphenolic content in relation to the treatment applied on the buds of the 'Ralli' variety

	Treatments ('Ralli')									
	Control	Garlic	DA	DB	EA	EB	TA	TB	SEM	P-Value
Procyanidin B1	737.7 ^a	889.5 ^{bc}	865.9 ^b	1057.9 ^e	947.9 ^{cd}	1190.4 ^f	962.3 ^d	1198.6 ^f	15.68	<0.001
Procyanidin B2	653.1 ^a	752.3 ^{bc}	701.2 ^{ab}	955.7 ^e	825.2 ^d	1116.9 ^f	789.3 ^{cd}	1012.9 ^e	15.48	<0.001
Catechin	385.2 ^a	445.2 ^b	442.9 ^b	602.7 ^c	488.3 ^b	696.7 ^d	487.6 ^b	675.6 ^d	13.17	<0.001

Chlorogenic acid	141.2 ^a	155.5 ^{ab}	158.2 ^b	204.1 ^d	179.4 ^c	231.4 ^e	163.1 ^b	202.2 ^d	3.62	<0.001
Vanillic acid	56.96 ^a	66.08 ^{abc}	65.51 ^{ab}	87.32 ^d	75.39 ^c	106.4 ^e	73.75 ^{bc}	100.4 ^e	2.22	<0.001
Epicatechin	1206.5 ^a	1386.8 ^b	1416.7 ^b	1660.3 ^d	1510.9 ^c	1872.1 ^f	1506.4 ^c	1769.6 ^e	19.9	<0.001
Piceid	67.67 ^a	75.65 ^b	75.21 ^b	103.5 ^d	83.69 ^c	117.8 ^e	83.69 ^{bc}	117.8 ^e	1.71	<0.001
Rutin	1160.9 ^a	1334.4 ^b	1405.2 ^b	1763.6 ^c	1436.24 ^b	2057.8 ^d	1642.1 ^c	2149.9 ^d	36.61	<0.001
o-coumaric	92.93 ^a	115.8 ^b	121.1 ^{bc}	179.8 ^d	141.1 ^c	195.2 ^{de}	140.7 ^c	209.2 ^e	4.69	<0.001
Resveratrol	111.7 ^a	136 ^b	136.1 ^b	189.3 ^d	150.2 ^{bc}	229.6 ^e	164.2 ^c	237.5 ^e	4.31	<0.001
ε-viniferin	244.6 ^a	281.2 ^{bc}	276.1 ^b	345.1 ^e	303.3 ^{cd}	405.4 ^f	314.2 ^d	404.2 ^f	5.35	<0.001
Quercetin	169.3 ^a	195.6 ^b	197.6 ^b	246.9 ^c	219.8 ^b	271.9 ^{cd}	214.5 ^b	293.7 ^d	5.82	<0.001
Luteolin	114.4 ^a	117.4 ^a	136.5 ^b	147.6 ^{bc}	143.7 ^{bc}	155.9 ^{cd}	151.4 ^{cd}	162.8 ^d	3.13	<0.001

380 Values are the means ($\mu\text{g g}^{-1}$ dw) of triplicates. Values in the same line with different superscript (a - f)
381 are significantly different at $P < 0.05$. The results are expressed as μg equivalent per g of dry weight
382 ($\mu\text{g g}^{-1}$ dw)

383

384 As mentioned earlier, the variations in polyphenolic compounds of latent buds
385 during their annual growth cycle seems to be related to hydrogen peroxide (Li et al.,
386 2011), but the combination of a cinnamic acid pretreatment and a cold treatment
387 further enhances the antioxidant activities. Proline and total polyphenolics content
388 increased under chilling stress (Shima et. al., 2017). Acclimation to low temperature
389 has been shown to increase concentrations of polyphenolic compounds in several
390 fruit tree species including the grapevine (Said et al., 2014; Karimi and Ershadi, 2015)
391 and pistachio (Pakkish et al., 2009).

392 All authors cited above showed that, in association with cinnamic acid, cold increases
393 H_2O_2 levels. That result seems to be in agreement with several studies dealing with
394 the involvement of the H_2O_2 in response to attacks of the abiotic environment
395 (Ashraf and Harris, 2004): it is quite likely that H_2O_2 acts as a messenger triggering a
396 modification of ionic flux or a production of secondary messengers such as salicylic
397 acid.

398

399 *Principal Component Analysis (PCA)*

400 PCA transforms the original data set, all measurements included, into a smaller set of
401 uncorrelated new variables (Principal Components, where eigenvalues are >1).
402 When performed on the polyphenolics studied, the PCA produced two (2)
403 components, in declining order of importance, which accounted for and explained

63.95% of the total variability between and among the different polyphenolics (Table 5).

Table 5: Principal components (PC) of the polyphenolics evaluated

Component		Percent of variance	Cumulative percentage
Number	Eigenvalue		
1	6.23	48.24	48.24
2	2.01	15.71	63.95

Polyphenolics compounds are presented in Figure 1 as a function of both the first and second principal components (PC). The first principal component (PC1) accounted for 48.24% of the total variability and was defined by polyphenolic compounds catechin, chlorogenic acid, epicatechin, o-coumaric acid, piceid, procyanidin B1, procyanidin B2, rutin, vanillic acid, and ϵ -viniferin. The fact that all compounds were located at a distance from the axis origin, suggests that they were well represented by PC1 and were placed close together on the positive side of the PC1 to indicate a strong, positive correlation. The second principal component (PC2) explained another 15.71% of the total variability and was defined by luteolin, quercetin, and resveratrol which were also placed close together on the positive side of PC2, to indicate that they positively correlated with one another. In contrast, luteolin did not correlate with ϵ -viniferin, catechin, nor did it do so with any of the remaining polyphenols.

424

425

426 **Figure 1:** Principal Component Analysis: plot of the polyphenols in relation to the first two

Figure 1: Principal Component Analysis: plot of the polyphenols in relation to the first two principal components (PC1 & PC2).

427 principal components (PC1 & PC2).

428

429 The first component contains the measurements gauging catechin, chlorogenic acid,

430 epicatechin, o-coumaric acid, piceid, procyanidin B1, procyanidin B2, rutin, vanillic

431 acid, and ϵ -viniferin. As to the second component, it gauges quercetin, resveratrol,
432 and luteolin. According to the PCA analysis for the purposes of the present study,
433 luteolin increased in contrast to catechin and ϵ -viniferin, as shown in Figure 1.

434

435 **Conclusions**

436

437 The results of the present study showed that chemically-induced stress conditions,
438 such as the ones caused by the application of chemical regulators which affect bud
439 dormancy release and advance budbreak, also affect the content of polyphenolic
440 compounds. Moreover, based on the experimental design that was implemented
441 through (a) the different applications taking place on specific days and (b) the cane
442 sampling taking place after a specific number of days had elapsed, the results
443 confirmed, for the first time in such studies, that variations in the levels of the
444 polyphenolic compounds identified do exist. Taking into consideration the fact that
445 polyphenolic compounds are essentially involved in the resistance response to
446 chilling stress, the variations in their levels constitute a first indicator of how much
447 stress the latent buds actually undergo.

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