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Polyphenolic diversity in *Vitis* sp. leaves

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ABSTRACT

We identified and quantified the main constitutive polyphenolic compounds of the leaves of seven *Vitis* species and of one interspecific cross, analysing leaf blades and veins separately, to spread light on the strategic localization of polyphenols in leaf tissues. To the best to our knowledge, the main leaf constitutive polyphenols of *V. candicans*, *V. coignetiae*, *V. vinifera sylvestris* and Börner were never described. *V. riparia* and *V. rupestris* (belonging to the same botanical series of *Ripariae*) displayed similar flavan-3-ol and dimeric proanthocyanidin concentration and similar percentage incidence of caftaric acid over total phenolic acids. *V. riparia* distinguished respect to the other genotypes for its high flavonol content, the highest percentage incidence of myricetin derivatives and an important diversification in the type of accumulated flavonol. *V. berlandieri* (series *Cinereae*) and Börner (hybrid of *V. riparia* x *V. cinerea*) accumulated low amounts of flavonol-glucosides comparing to the other species, but they showed a wide profile diversification, as well. However, it was *V. v. sylvestris*, the wild ancestor of *Vitis vinifera* subsp. *sativa* that displayed the widest flavonol profile diversification. The differences in the flavonol profile could be related to the genus *Vitis* evolution: in fact, with domestication, the flavonoid biosynthetic pathway underwent a progressive simplification; for this, the highest flavonol diversity found in *Vitis v. sylvestris* is probably a demonstration of its reduced or nil level of domestication. *V. amurensis*, known for its cold tolerance and resistance to downy mildew, anthracnose and white rot, markedly differentiated respect to the other genotypes, for its high concentration of polyphenols, particularly of vein flavonols and flavanonols. Moreover, *V. amurensis* leaves generally presented a constantly high concentration of constitutive polyphenols throughout the season that probably contributes to protect against adverse environmental condition. The abundance of polyphenols in *V. amurensis* leaves emphasizes that this species is a source of natural bioactive compounds that could find application for nutraceutical and pharmacological uses. *V. berlandieri* and Börner markedly distinguished respect to the other studied species for their exclusive capability to accumulate flavones (mainly orientin, isoorientin, vitexin and isovitexin) in blades and in veins, in considerable amounts.

Knowledge about these subjects could contribute to shed light on the identification of species-related molecules involved in the plant-defense mechanisms, to the chemotaxonomy of the genus *Vitis*, to the possibility of identifying specific natural bioactive compounds to use in plant-based preparation for nutraceutical, cosmetic, feed/food-additive purposes.

KEYWORDS: *V. amurensis, V. candicans, V, riparia, V. rupestris, V. berlandieri, V. coignetiae, V. vinifera sylvestris*, Börner.

1. INTRODUCTION

The genus *Vitis* comprises more than 60 species, mostly inhabiting temperate regions. The most known and economically important one is the western Eurasian species *Vitis vinifera* subsp. *vinifera*, which ancestor is the wild *Vitis vinifera* subsp. *sylvestris*, naturally occurring in Europe, Middle East and Northern Africa. Up to thirty grapevine species are native of East Asia and North America, areas that host a wide diversity of *Vitis* species (Wan et al., 2013).

Vitis vinifera is susceptible to many pests. *Phylloxera* (*Daktulosphaira vitifoliae*), downy and powdery mildew (caused by *Plasmopara viticola* and *Erysiphe necator*, respectively), imported almost simultaneously from North America to Europe in the XIX century, are among those causing important economic loss in viticulture. Viticulturists, technicians and researchers overcame the problem tied to *vinifera* sensitiveness to *Phylloxera* by using American species or their hybrids as rootstocks. Furthermore, the introduction of downy and powdery mildew pushed to develop breeding programs based on the constitution of interspecific crosses *vinifera*-American species that, together with Asian species, were disease-tolerant or resistant due to their coevolution with the pathogen. *Vitis* species such as *V. riparia*, *V. rupestris*, *V. californica* and *V. amurensis* are known as highly tolerant to downy mildew (Gómez-Zeledón et al., 2013; Jürges et al., 2009); *V. riparia*, *V. munsoniana*, *V. candicans*, *V. rotundifolia* as highly resistant to powdery mildew (Staudt, 1997; Wan et al., 2007) and *V. rotundifolia* (subspecies *Muscadinia*) and *V. arizonica* as resistant to Pierce's disease (Ruel and Walker, 2006). Besides, some species distinguish for their tolerance to environmental stressors thanks to their origin: it is the case of *V. amurensis*, native of cold areas in northeastern China and Russian Siberia, that is highly frost tolerant (Zhang et al., 2012).

To prevent disease or damage caused by biotic or abiotic stresses, plants employ a complex defense system, which involves a broad spectrum of physical and biochemical changes. Biochemical resistance, such as preformed defenses, has evolved to face environmental stressors and is also related, among others, to secondary metabolites, including polyphenols (reviewed in Llorens et al., 2017 and in Dixon, 2001). *Vitis vinifera* is known for its abundance and richness in polyphenols that have been and

currently are widely studied in the berries due to their implications in the technology of winemaking and in the quality of derived wines. Thousands of scientific studies were devoted to define the polyphenolic composition of *Vitis vinifera* berries, and, to a much lesser extent, to vegetative organs; oppositely, less is known about other *Vitis* species polyphenolic composition, particularly as to vegetative organs. Moore and Giannasi (1994) described the qualitative composition of flavonols and flavones in leaves of some North American *Vitis* species. Flavonol profiles were described also in *V. amurensis* (Hmamouchi et al., 1996) and *V. rotundifolia* (Pastrana-Bonilla et al., 2003) leaves. Main phenolic compounds were also analyzed in *V. labrusca* leaves (Dani et al., 2010; Dresch et al., 2014). Chen et al. (2018) reviewed *V. amurensis* polyphenolic composition and related pharmacological properties. At present, the scientific interest about *Vitis* sp. polyphenolic composition is at least dual: to investigate the health beneficial effects of grapevine leaves as a possible source of natural bioactive compounds, that can be used in nutraceutical and pharmaceutical applications, and to deepen knowledge about their involvement in grapevine defense mechanisms. Different *Vitis* species might display different metabolic pathways and/or different gene regulations, resulting in the production of specific classes of bioactive polyphenols, or of individual molecules. Knowledge about these subjects could contribute to shed light on the identification of species-related molecules involved in the plant-defense mechanisms, to the chemotaxonomy of the genus *Vitis* and to the possible identification of specific molecules to use in specific plant-based preparation for nutraceutical, cosmetical, nutritional purposes.

The aim of the present study was to identify the main constitutive polyphenolic compounds in the leaves of seven *Vitis* species and of one interspecific cross (the rootstock Börner, *V. riparia x V. cinerea;* Figure 1). To the best to our knowledge *V. candicans*, *V. coignetiae*, *V. v. sylvestris* and Börner main leaf constitutive polyphenols were never described. Leaf blades and veins were analysed separately to spread light on polyphenol strategic localization in leaf tissues, to contribute to explain the grapevine-pathogen interaction in specific biological systems.

2. MATERIALS AND METHODS

2.1 Plant Material. Four American (*V. candicans*, *V. riparia*, *V. berlandieri* and Börner), two Asian (*V. amurensis*, *V. coignetiae*) and one Eurasian (*V. v. sylvestris*) grapevine genotypes were studied (Table 1; Figure 1). All the studied accessions were previously checked for their trueness to type by morphology and molecular markers. The studied *Vitis* genotype leaves were collected in the collection vineyard of DISAFA, University of Turin, Grugliasco (Piedmont, Italy), sampled five times during the season: 1 = 28th of May (148 day of the year - DOY), 2 = 22nd of June (DOY 173), 3 = 14th of July (DOY 195), 4 = 3rd of August (DOY 215), 5 = 28th of August (DOY 214), in 2015. The collection vineyard was managed routinely during spring and summer as already described (Kedrina-Okutan et al., 2018) (Kedrina-Okutan et al., 2018). One field parcel was generally constituted by four consecutive plants used to collect the leaves; leaves were detached between the fourth and seventh node of the main shoots from the west side of the row. Pools of 15 adult and healthy leaves (visual evaluation) were collected at each sampling time and divided into three replicates. After sampling, leaves were immediately transported to the laboratory for further analyses.

2.2 Reference Compounds and Reagents. Orientin**,** isoorientin, astilbin and caftaric acid were purchased from Sigma-Aldrich S.r.l. (Milan, Italy). Vitexin, *trans*-fertaric acid and *trans*-coutaric acid were purchased from Phytolab (Vestenbergsgreuth, Germany). (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate, proanthocyanidin B_1, proanthocyanidin B_2, quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, kaempferol 3-*O*-glucoside, kaempferol 3-*O*-glucuronide, myricetin 3-*O*-glucoside and isorhamnetin 3-*O*-glucoside from Extrasynthèse (Genay, France). Folin−Ciocalteu reagent and tartaric acid were purchased from Merck (Darmstadt, Germany); sodium sulfate and sodium metabisulfite were purchased from BDH Laboratory Supplies (Poole, England).

2.3 Measurement of Dry Matter. Leaf tissue dry matter was measured three times during the season at DOY 186, DOY 200 and DOY 241. Leaf veins and blades were separated and dried inside an oven for 72 hours at constant temperature of 110 ºC. The weight differences after exsiccation were measured, and results were expressed as percentage of dry weight over fresh weight.

2.4 Sample Extraction for Polyphenol Analyses. The 15 collected leaves were divided into sub-samples of five, and veins and blades were immediately separated. Two grams of tissue were randomly chosen form the three sub-samples and extracted in a hydroalcoholic buffer (pH = 3.9, 40% ethanol, 22 mL L^{-1} of 1 N NaOH, 5 g L^{-1} of tartaric acid, 2 g L^{-1} of sodium metabisulfite). For sample homogenization and polyphenol extraction, an Ultraturrax dispersing machine (IKA, Staufen, Germany) was used for around 1 min setting the speed at 10 000 rpm followed by 10 min of centrifugation at 4000 rpm. The supernatant was separated, and the pellet was resuspended with the same buffer; the resuspension was kept in the dark for 30 min and then centrifuged again. The two supernatants were combined and brought to a final volume of 50 mL. Obtained leaf extracts were stored at -20 ºC until further analysis.

2.5 Measurement of Total Polyphenols. The method of Singleton et al. (1999) was used and results were expressed as gram of (+)-catechin equivalents (CE) per kg of blades/veins dry weight (DW). Briefly, 100 μL of leaf extract were mixed with 5 mL ultrapure water and 1 mL of Folin-Ciocalteu reagent was added, mixed and incubated at room temperature for 5 min. After incubation, 4 mL of 10% Na_2CO_3 and ultrapure water were added until final volume of 20 mL. The mixture was incubated in the dark for 90 min and the absorbance was read at 760 nm by UV/Vis spectrophotometer (PerkinElmer, Lambda 25, Beaconsfield, Bucks, UK).

2.6 Analysis of individual polyphenols.

2.6.1 Sample preparation. Prior to the chromatographic analysis, the extracts were diluted with 1 M phosphoric acid 90/10 v/v and passed through 0.20 µm membrane filter GHP Acrodisc (PALL Italia, Buccinasco, Milan, Italy) (Di Stefano and Cravero, 1992).

Individual phenolic compound analysis by HPLC-ESI-MS/MS. Chromatographic separation was performed by HPLC-DAD system (HPLC 1100, Agilent Technologies, U.S.) equipped with column Luna C-18 150 x 2 mm (Phenomex Aschaffenburg, Germany). Mobile phase solvent A was water with formic acid 0.1% and solvent B was methanol with formic acid 0.1%; the gradient program for solvent B was the following: 0-30 min 0-50 % B; 30-35 min 50-100 % B; 35-50 min 100 % B and back to initial

conditions from 50 to 55 min, followed by 10 min of isocratic flow. Injection volume was 5 µL, flow rate 200 µL min^{-1} and individual phenolic compounds were detected at 280, 320, and 370 nm. For the mass spectrometry analyses, a Bruker Daltonics esquire 3000^{plus} ion trap spectrometer (Bruker Daltonics, HB, Germany) equipped with electrospray (ESI) was used, operating at positive and negative modes. The scan mode was between 100-800 m/z, with a scan resolution of 13 000 m/z/s until the ICC target reached either 20 000 or maximum accumulation time of 200 ms. The MS instrument operated with nitrogen as drying gas at a temperature of 330 °C (flow rate of 9 L min^{-1}). Ionization voltage of the electrospray capillary source was 4000 V and tandem MS was carried out using helium as the collision gas (4.21 x 10^{-6} mbar) with 1 V collision voltage. MS identification of metabolites was according to mass spectra, product ion spectra, retention time and confirmed with authentic standards and published data.

2.6.2 Individual phenolic compound quantification by HPLC-DAD. The quantification of phenolic compounds was performed by HPLC-DAD according to previously published methods (Di Stefano and Cravero, 1992; Ferrandino and Guidoni, 2010). Stationary phase column was Licrosphere 100 RP5 µm) packed into LiChroCART 250-4 (25 × 0.4 cm ID) HPLC-Cartridge (Merck KGaA, Germany) with guard column (LiChroCART 4-4). Mobile phase solvent A was phosphoric acid 10^{-3} M and solvent B was pure methanol. Run time was 50 min, temperature 25 °C and DAD peaks were detected at 280 nm, 320 nm and 360 nm. Compounds were identified based on compliance with data obtained from available pure standards and quantified based on standard curve constructed per each individual molecule. Among flavanonol, as exclusively dihydroquercetin-rhamnoside (astilbin) was available as commercial standard, semi-quantification of individual compounds was carried out using the astilbin standard curve. The average flavonol profile of individual genotype was calculated averaging results of five sampling dates.

2.7 Statistical analysis.

All data are averages of three biological replicates and standard errors. The analysis of variance was performed by one-way ANOVA with IBM SPSS Statistics software program version 24.0 for Windows

(SPSS Inc., Chicago, IL). In case of significant differences ($P \leq 0.05$), means were compared by Tukey-b post-hoc test. Results related to leaf blade and vein polyphenolic composition taken separately were used to run a series of principal component analysis (PCA); correlated variables were progressively excluded, when this correlation had a biological meaning. Species and dates of sampling were separated on the basis of specific classes of polyphenols (i.e. variables). PCA analysis was performed by SAS 9.4 for Windows (SAS Institute Inc., Cary, NC, US).

Table 1. Origin and botanical sub-generic series of the studied genotypes.

Plant name	Abbreviation	Origin	Series[a]
Vitis candicans	CAN	American	*Candicansae*
Vitis riparia cv Gloire de Montpellier	RIP	American	*Ripariae*
Vitis rupestris cv du Lot	RUP	American	*Ripariae*
Vitis berlandieri	BER	American	*Cinereae*
Börner *(V. riparia* x *V. cinerea)*	BOR	American	*Ripariae X Cinereae*
Vitis coignetiae	COI	Asian	*Lambruscae*
Vitis amurensis	AMU	Asian	*Flexuosae*
Vitis vinifera subsp. *sylvestris*	SYL	Eurasian	*Viniferae*

[a]Series were defined according to Galet (1988).

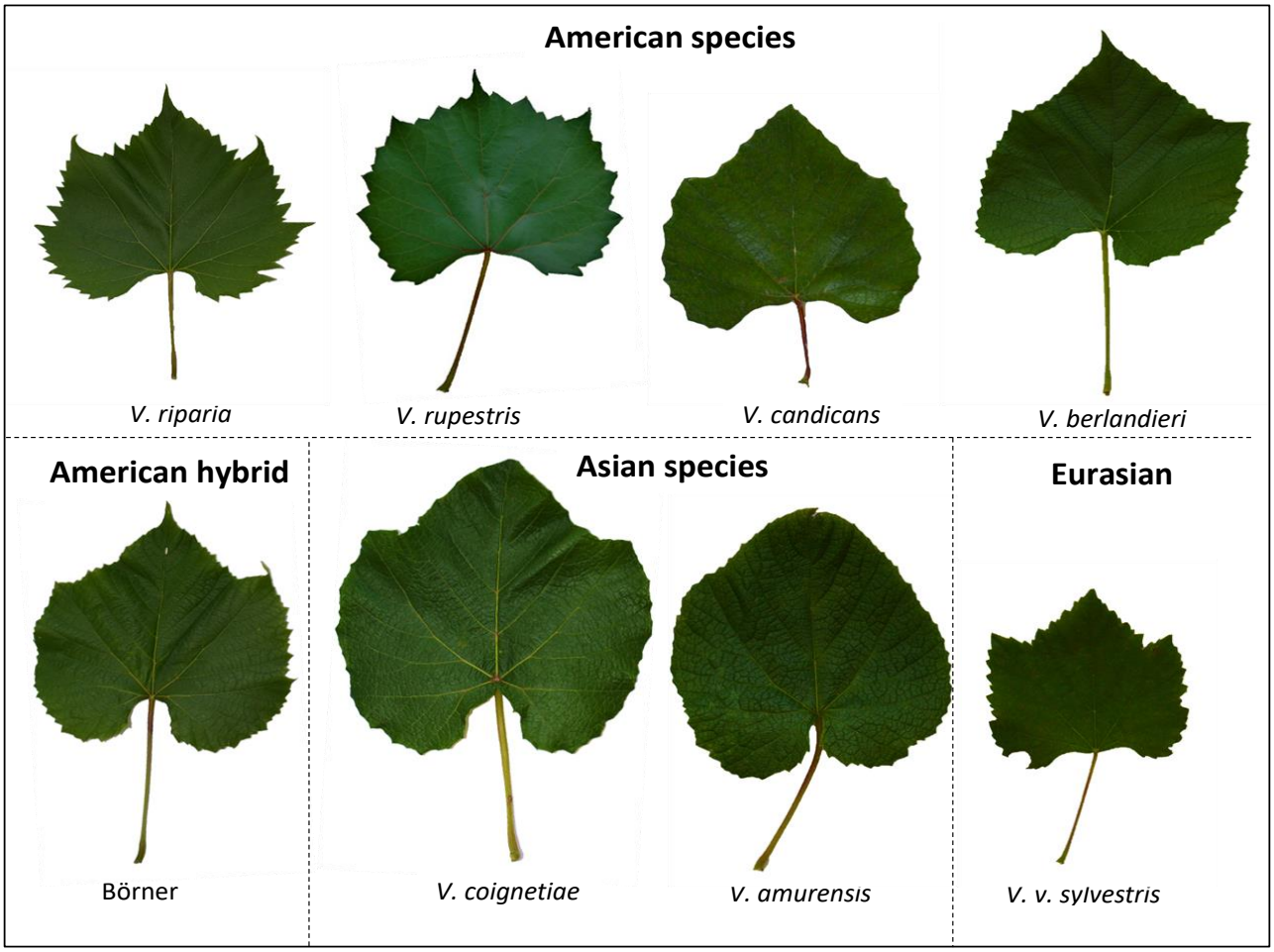

Figure 1. Leaves of eight *Vitis* genotypes.

3. RESULTS

Significant differences of water content occurred among *Vitis* species at the same picking time (Table 2); however, differences did not exceed 7% in blades and 8% in veins. Within the species, water content evolution during the vegetative season was not higher than 6%; in blades of *V. candicans*, *V. v. sylvestris*, Börner and *V. coignetiae* dry matter (DM) content changes during the season were never significant. The comparison between averages of all analyzed grapevine blades and veins showed that dry matter content in veins was around 10% lower than in blades. Concentrations of compounds were calculated and expressed as amounts per dry matter, even though leaf extracts were prepared from fresh leaves: this choice was done to minimize the well detailed polyphenol analysis perturbations due to losses or to chemical alterations during preparation (Abascal et al., 2005).

Table 2. Dry matter (%) in leaves *of Vitis* genotypes during the vegetative season [a]

	DOY 186		DOY 200		DOY 241		date
		#	Blades	#		#	Sampling dates
AMU	32.09 ± 0.03a	e	34.43 ± 0.58b	c	35.29 ± 0.23b	bc	*
CAN	29.71 ± 0.30	cde	29.68 ± 0.18	ab	28.94 ± 1.63	a	ns
RIP	27.78 ± 0.69a	ab	28.05 ± 0.79a	a	31.95 ± 1.21b	ab	*
RUP	27.35 ± 0.75a	a	27.16 ± 0.80a	a	33.51 ± 1.01b	b	**
BER	31.45 ± 0.18a	de	31.86 ± 1.07a	bc	37.57 ± 0.61b	c	**
COI	29.59 ± 0.43a	bc	32.09 ± 0.70b	bc	32.93 ± 0.53b	ab	*
SYL	31.39 ± 0.62	de	29.70 ± 0.83	ab	31.76 ± 0.61	ab	ns
BOR	30.57 ± 0.66	de	31.49 ± 0.74	bc	32.88 ± 0.35	ab	ns
average	29.99 ± 0.37		30.56 ± 0.51		33.10 ± 0.56		
species		**		**		**	
			Veins				
AMU	19.01 ± 0.42a	ab	22.61 ± 0.47b	bc	22.32 ± 0.33b	b	**
CAN	23.14 ± 0.70b	b	23.61 ± 0.99b	c	19.32 ± 0.60a	a	*
RIP	21.21 ± 0.71ab	b	20.52 ± 0.52a	ab	22.88 ± 0.21b	bc	*
RUP	19.23 ± 1.43a	ab	20.02 ± 0.52a	ab	25.13 ± 0.34b	cd	**
BER	21.37 ± 0.40a	ab	23.08 ± 1.12a	bc	26.30 ± 0.58b	d	*
COI	17.82 ± 0.53	a	18.89 ± 0.72	a	19.27 ± 0.66	a	ns
SYL	21.22 ± 0.47a	ab	23.74 ± 0.15b	c	27.28 ± 0.48c	d	**
BOR	20.59 ± 0.45a	ab	20.26 ± 0.33a	ab	23.88 ± 0.81b	bc	**
average	20.45 ± 0.38		21.59 ± 0.42		23.30 ± 0.60		
species		*		**		**	

[a] Results are expressed as means ± standard errors (SE; n=3). Different small letters in each individual row after averages ± SE represent statistical differences among sampling dates, within the same species. In columns individuated with #, different letters represent statistical differences among genotypes, within the same tissue and date. General statistical differences among sampling dates and species were assessed by a post-hoc Tukey-b test for $P \leq 0.05$ (*), $P \leq 0.01$ (**); ns – not significant. Day of the year (DOY) refers to 5th of July (DOY 186); 19th of July (DOY 200); 29th of August (DOY 241).

The total polyphenol (TP) content ranged from 60.2 to 165.9 g kg^{-1} DW in blades and from 28.6 to 130.1 g kg^{-1} DW in veins. *V. amurensis* blades (Figure 2) displayed higher TP concentration during the entire vegetative season compared to the other genotypes. TP was on average about 1.5 times lower in veins than in blades. TP accumulation trend was different among genotypes: in *V. riparia*, the accumulation trends in veins and blades were similar, whereas in *V. candicans*, TP concentration increased in blades and decreased in veins during the season.

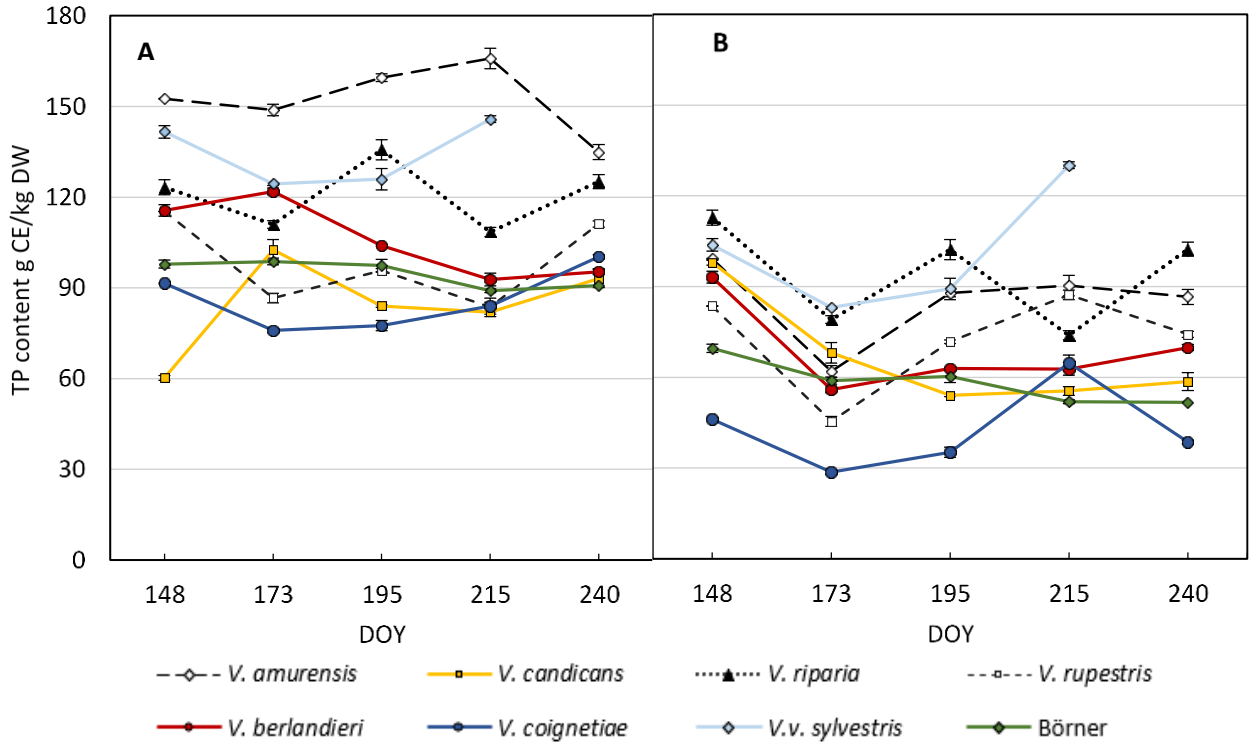

Figure 2. Total polyphenol (TP) accumulation in leaves of eight *Vitis* genotypes during the vegetative season; A = blades and B = veins. Means of three replicates ± SE.

3.1 Phenolic Compound Accumulation in *Vitis sp*. Leaves. Twenty phenolic compounds were identified in the two Asian species, 27 in the four American species and in the hybrid, and 18 in *V. v. sylvestris*. The detected compounds belonged to the groups of flavonols, phenolic acids, flavan-3-ols, proanthocyanidins, flavanonols and flavones. Anthocyanins were exclusively detected in trace amounts or in extremely low concentrations (data not shown) and, in line with what we previously found in varieties of *V. vinifera sativa* (Kedrina-Okutan et al., 2018), in healthy leaves they were found exclusively in veins.

3.1.1 Flavonols.

The concentration of flavonol glycosides ranged from 3.6 to 20.6 g kg^{-1} DW in blades and from 0.8 to 7.7 g kg^{-1} DW in veins (Figure 3); they were the most abundant phenolic compounds in leaves. We detected a net separation among genotypes based on their ability to accumulate flavonol glycosides during the season. A first group included *V. amurensis* and *V. riparia*, both species displaying the highest

total flavonol concentration, with a peak at DOY 196 in *V. riparia* and at DOY 215 in *V. amurensis* (Figure 3A). A second group with medium flavonol concentration included *V. v. sylvestris* and *V. rupestris* with a stable concentration trend during the vegetative season. A third group included the remaining species, displaying relatively low total flavonol concentration and slightly decreasing trends during the examined period. Similar results were found in veins: genotypes of the first two groups displayed higher total flavonol concentration and genotypes of the third group lower concentration with decreasing trend during the season (Figure 3B). The comparison between the two compartments within each genotype showed that total flavonol concentration was always higher in blades than in veins. The prevalent flavonol was quercetin 3-*O*-glucuronide, ranging between 60% and 92% of all the detected flavonols (Figure 4; Supplementary Table 1A,B). The most complex flavonol profile was found in *V. v. sylvestris* leaves accumulating seven out of the eight identified flavonol glycosides and trace amount of kaempferol 3-*O*-glucoside (Figure 4). Kaempferol 3-*O*-rhamnoside was exclusively found in *V. v. sylvestris* that also accumulated significant amounts of quercetin 3-*O*-rhamnoside. The simplest flavonol profile was found in *V. amurensis* and *V. candicans* that exclusively accumulated quercetin and kaempferol glucuronides and glucosides. In *V. berlandieri* and in Börner, no kaempferol glycosides were detected.

3.1.2 Phenolic acids.

Total phenolic acid content in blades ranged from 3.2 to 10.2 g kg^{-1} dry weight and showed different accumulation trends among genotypes. In Asian species, *V. amurensis* and *V. coignetiae*, total phenolic acid concentration fell during the vegetative season. In American species, concentration firstly increased and then it started to fall between July and beginning of August (DOY 173-215), whereas in *V. v. sylvestris*, concentration decreased between the first and the second sampling date and then rose from the end of June (DOY 173) to the beginning of August (DOY 215) (Figure 5A). In leaf veins, phenolic acid content showed a significant drop between the first and the second sampling date in all genotypes, followed by a relatively steady period (Figure 5B). Total phenolic acid concentration in veins was lower than in blades in each individual species, except at the first sampling date in *V. candicans*, *V.*

riparia and *V. rupestris* when concentration in veins was higher than in blades. Hydroxycinnamoyl tartaric acids were the prevalent phenolic acids in leaves, and, based on their characteristic UV maximum absorbance around 320 nm and mass spectra, they were identified as *cis*- and *trans*-forms of caftaric, coutaric and fertaric acid (Table 3). *Trans*-forms were always prevalent over *cis*-forms The main phenolic acid in leaves was *trans*-caftaric acid, which comprised up to 90% followed by *trans*-coutaric and *trans*-fertaric acid (Figure 5C, Supplementary Table 2A, B). On the contrary, in *V. rupestris* and *V. riparia trans*-fertaric acid displayed higher concentration respect to *trans*-coutaric acid. Moreover, these two species accumulated an additional molecule, a coutaric acid isomer, that was tentatively identified through its pseudomolecular ion $[M-H]^-$ at *m/z* 295 and two product ions at *m/z* 163, 131 in MS^2. Low amounts of protocatechuic acid-glucoside were detected in *V. rupestris*, *V. riparia*, *V. amurensis* and Börner blades, with a slight increasing trend during the season (Supplementary Table 2A).

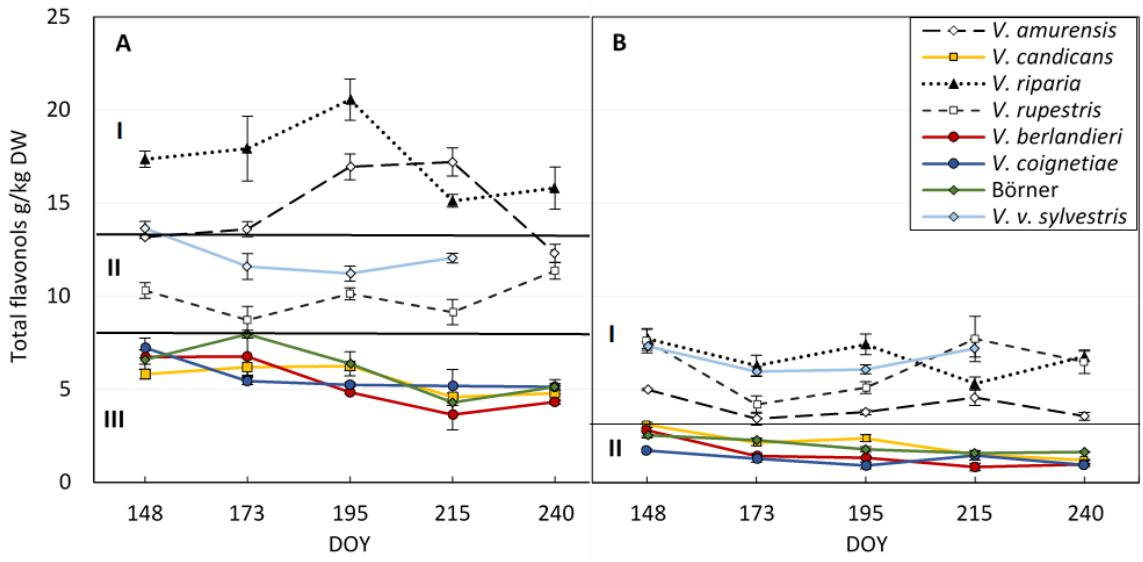

Figure 3. Total flavonol concentration in leaves of eight *Vitis* genotypes during the vegetative season; A = blades and B = veins. Means of three replicates ± SE.

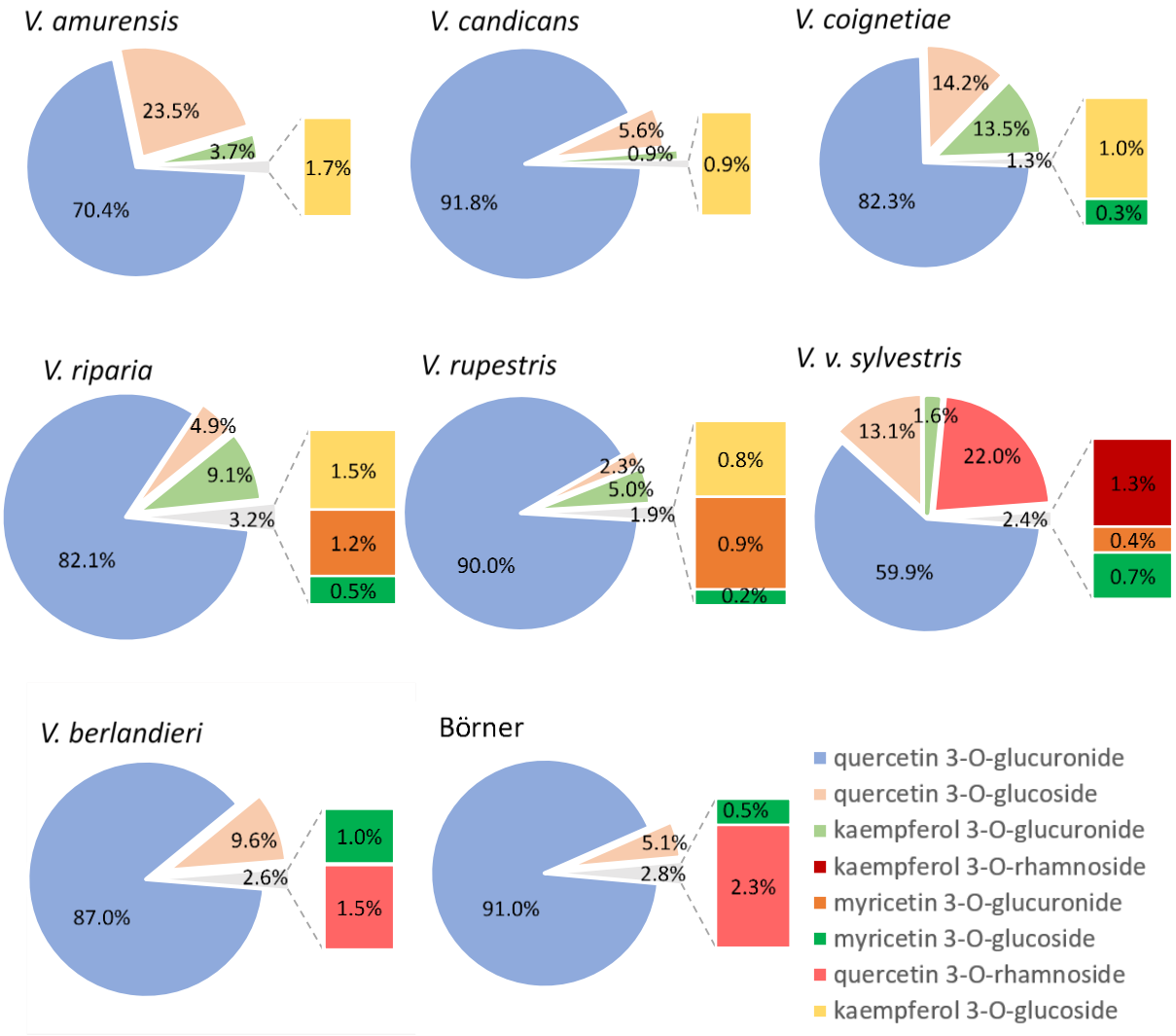

Figure 4. Flavonol profile of leaf blades of eight *Vitis* genotypes calculated as average of results obtained at five sampling times performed during the vegetative season.

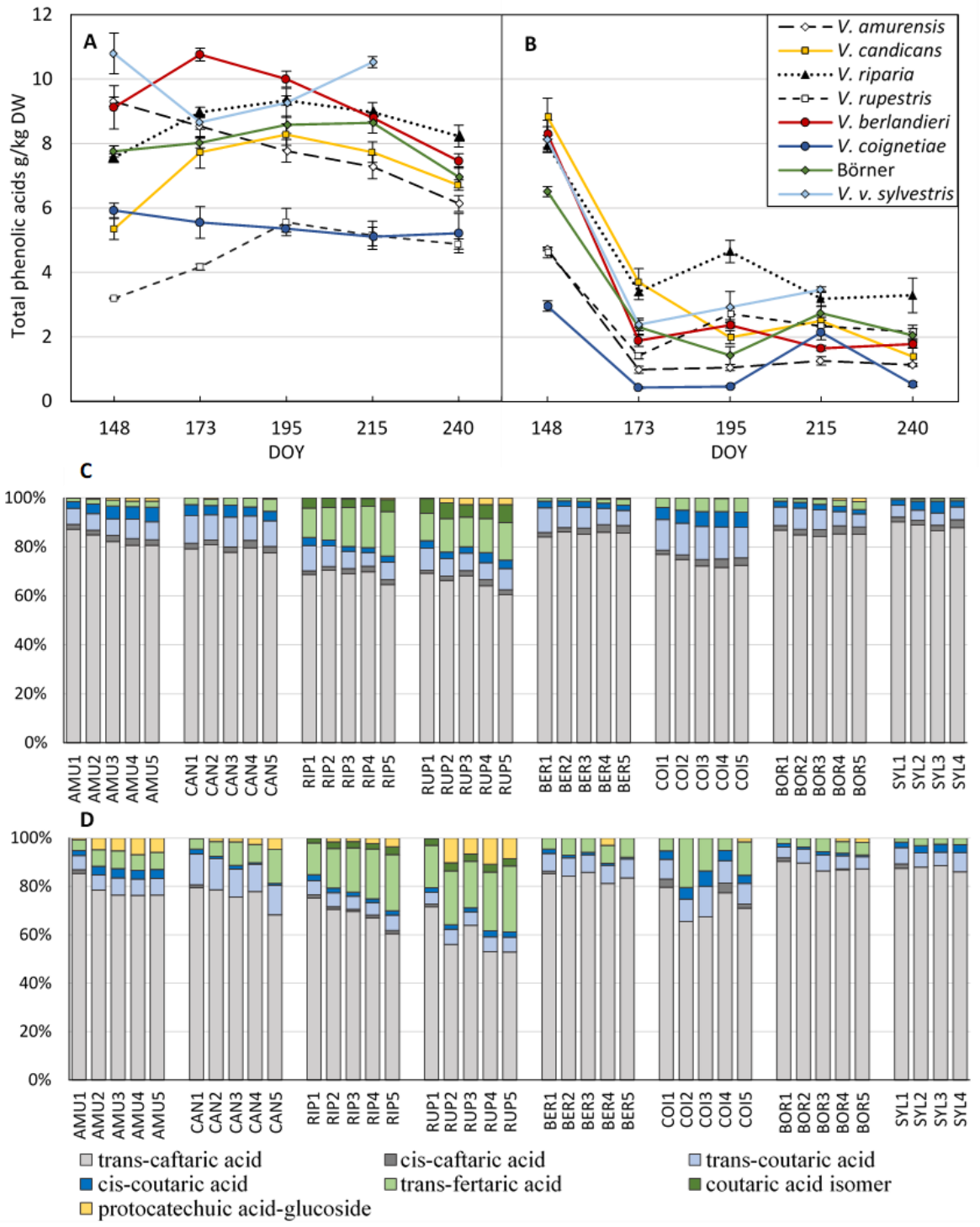

Figure 5. Accumulation of phenolic acids in *Vitis sp.* leaves during the vegetative season. Total phenolic acid concentration in blades (A) and veins (B); results are means of three replicates ± SE. Phenolic acid profile of leaf blades (C) and veins (D) during the season. For species acronyms see Table 1; numbers after the species acronyms refer to 1 = DOY 148 (28th of May); 2 = DOY 173 (22nd of June); 3 = DOY 195 (14th of July); 4 = DOY 215 (3rd of August); and 5 = DOY 240 (28th of August).

Table 3. Phenolic compound identification in leaf blades and veins of eight *Vitis* genotypes by HPLC-ESI-MS/MS

ID	Rt (±0.2 min)	$[M]^-$ (m/z)	$[MS^2]^-$ (m/z)	$[M]^+$ (m/z)	$[MS^2]^+$ (m/z)	compound identification
1	14.5	593	425, 407, 289	595	291	(epi)gallocatechin-(epi)catechin
2	16.5	315	153, 123			protocatechuic acid-glucoside
3	18.3	311	179			*cis*-caftaric acid
4	18.8	577	451, 407, 289			procyanidin B_3
5	19.9	311	179			*trans*-caftaric acid
6	21.8	289	245, 205, 179	291	273, 165, 123	catechin
7	22.5	295	163			*cis*-coutaric acid
8	23.0	577	451, 425, 407, 289	579	427, 409, 289	procyanidin B_1
9	23.5	295	163			*trans*-coutaric acid
10	23.8	729	577, 441, 289	731	579, 409	procyanidin dimer gallate
11	24.9	595	576, 385, 355			unknown proanthocyanidin
12	25.1	325	193			*trans*-fertaric acid
13	25.7	289	245, 205, 179	291	273, 165, 123	epicatechin
14	27.7	465	303, 285, 151			dihydroquercetin-hexoside
15	27.7	295	163, 131			coutaric acid isomer
16	29.2	615	481, 407, 359			unknown
17	30.8	447	393, 357, 327	449	413, 383, 329	luteolin 8-*C*-glucoside (orientin)
18	31.1	449	287, 269, 151			dihydrokaempferol hexoside
19	31.4	447	429, 357, 327, 285	449	431, 413, 383	luteolin 6-*C*-glucoside (isoorientin)
20	31.8	479	317	481	319	myricetin 3-*O*-glucoside
21	32.3	431	341, 311, 283	433	415, 367, 283	apigenin 8-*C*-glucoside (vitexin)
22	32.7	493	317	495	319	myricetin 3-*O*-glucuronide
23	33.7	449	303, 285, 151			dihydroquercetin-rhamnoside (astilbin)
24	33.8	431	413, 341, 311, 283	433	415, 367, 337	apigenin 6-*C*-glucoside (isovitexin)
25	34.2	447	285	449	287	luteolin-7-*O*-glucoside (luteoloside)
26	35.1	463	301	465	303	quercetin 3-*O*-glucoside
27	35.6	477	301	479	303	quercetin 3-*O*-glucuronide
28	37.1	447	301	449	303	quercetin 3-*O*-rhamnoside
29	37.2	447	285	449	287	kaempferol 3-*O*-glucoside
30	37.5	461	285	463	287	kaempferol 3-*O*-glucuronide
31	38.3	431	285	433	287	kaempferol 3-*O*-rhamnoside

[a]ID numbers correspond to peaks reported in Supplement Figure 1, 2.

3.1.3 Flavan-3-ols.

Important quantities of total flavan-3-ols were detected in grapevine leaves. The concentration of flavan-3-ols was rather similar between blades and veins, oppositely to flavonols and phenolic acids that prevalently accumulated in blades. Specifically, the concentration of flavan-3-ols ranged from 0.44 to 3.39 g kg^{-1} in blades and from 0.04 to 1.55 g kg^{-1} in veins (Figure 6A and B). In *V. amurensis* blades, the concentration of total flavan-3-ols was significantly higher respect to the other genotypes, and it

displayed a decreasing trend, so that at the end of the examined period the differences respect to the other genotypes were less marked. In *V. rupestris* flavan-3-ol concentration increased almost continuously during the entire vegetative season, oppositely to the other analyzed genotypes. The flavan-3-ol profile analysis showed that (+)-catechin was generally the prevalent flavan-3-ol, corresponding up to 73 % of total flavan-3-ols in blades (Figure 5C; Supplementary Table 3A) or the exclusive one in *V. coignetiae* veins (Figure 5D; Supplementary Table 3B). In the Asian species *V. amurensis,* (-)-epicatechin was the prevalent flavan-3-ol in blades, similarly to *V.v. sylvestris* and *V. rupestris* veins from the second sampling date onwards. Concentration of (-)-epigallocatechin showed an increasing trend during the examined season and in blades it accounted up to 38% by the end of the season. Interestingly, in veins of *V. coignetiae* and Börner, (-)-epicatechin was absent during the whole season, and in *V. candicans* it was exclusively detected at the first sampling date. The relative abundance of (-)-epicatechin gallate ranged between 4.7% and 14.9% and it was exclusively found in blades of *V. rupestris*, *V. berlandieri* and Börner.

3.1.4 Proanthocyanidins.

Proanthocyanidin concentration ranged from 0.21 to 3.09 g kg^{-1} in blades. Particularly high concentration was detected in *V. amurensis* at first four sampling dates and in *V. berlandieri* at first two sampling dates, followed by a sharp concentration fall (Figure 7A). In veins, PA exclusively accumulated in *V. amurensis* and *V. candicans* during the whole season and in some dates in four species, also in *V. coignetiae* and *V. v. sylvestris*. PA content was generally higher in blades than in veins, with the only exception of *V. amurensis* and *V. candicans* at DOY 148 (end of May) when vein PA content was higher respect to that of blades (Figure 7; Supplementary Table 4A,B). Procyanidin B_1 accumulated both in blades and veins. Additionally, in *V. amurensis*, *V. berlandieri* and in Börner blades and in *V. amurensis* veins, a procyanidin dimer gallate was tentatively identified by pseudomolecular ion $[M\text{-}H]^-$ at *m/z* 729; product ions at *m/z* 577, 441, 289 in MS^2 and characteristic UV maximum absorbance at 280 nm, in line with other report (Flamini, 2013). In *V. berlandieri* and Börner blades,

procyanidin dimer gallate accounted for around 50% of total PA, whereas in *V. amurensis*, it accounted for 20-25 % of total PA during the vegetative season.

Furthermore, by HPLC-ESI-MS/MS (epi)gallocatechin-(epi)catechin was tentatively identified in blades (pseudomolecular ion $[M-H]^-$ at *m/z* 593; product ions at *m/z* 425, 407, 289 in MS^2; Table 3), In addition, in *V. amurensis* blades, another B-type of procyanidin was identified, most likely procyanidin B_3 and one further proanthocyanidin that remains unknown (Table 3).

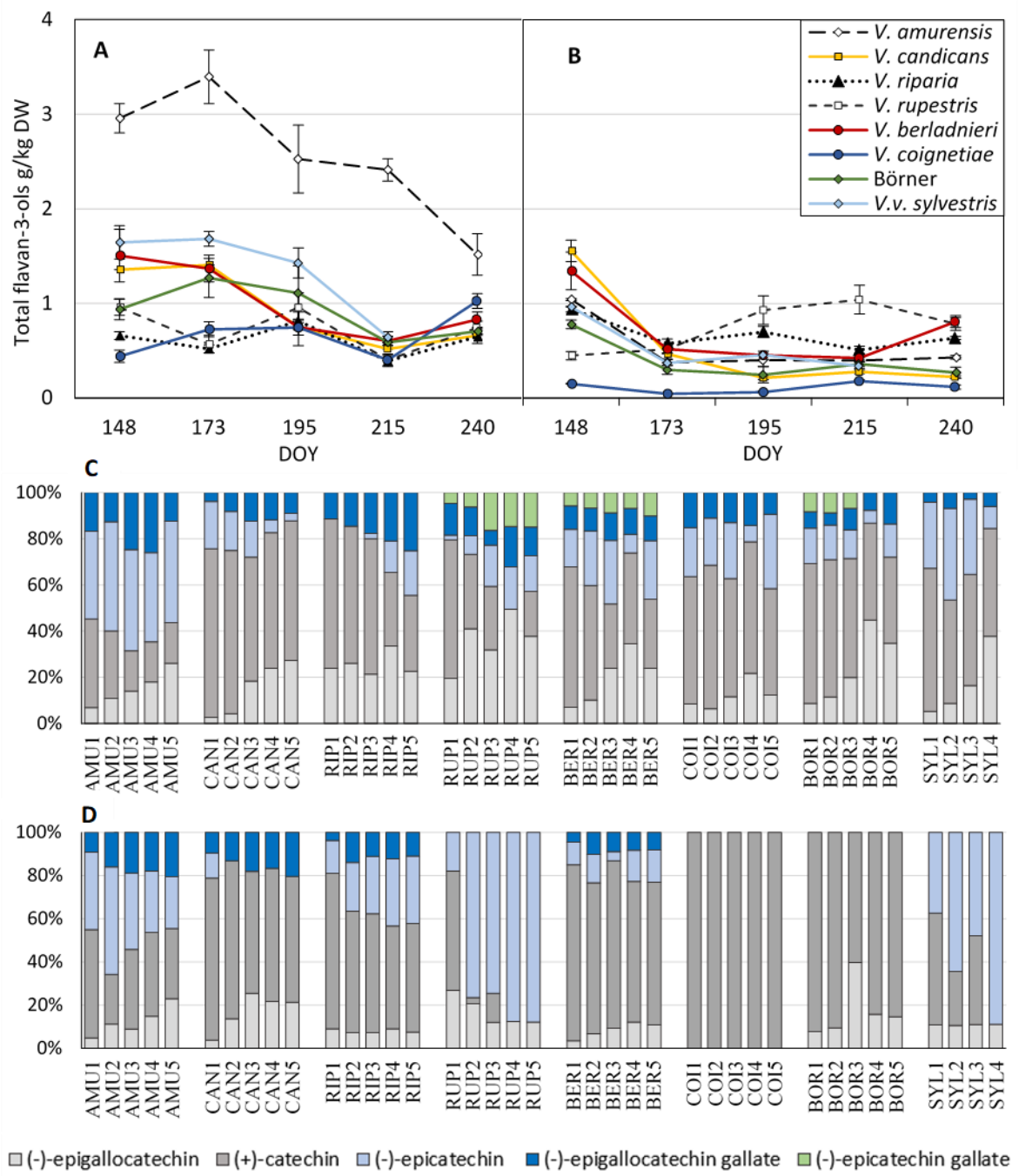

Figure 6. Accumulation of flavan-3-ols in *Vitis sp.* leaves during the vegetative season. Total flavan-3-ol concentration in blades (A) and veins (B); results are means of three replicates ± SE. Flavan-3-ol profile of leaf blades (C) and veins (D) during the season; see Figure 5 for genotype acronym specification.

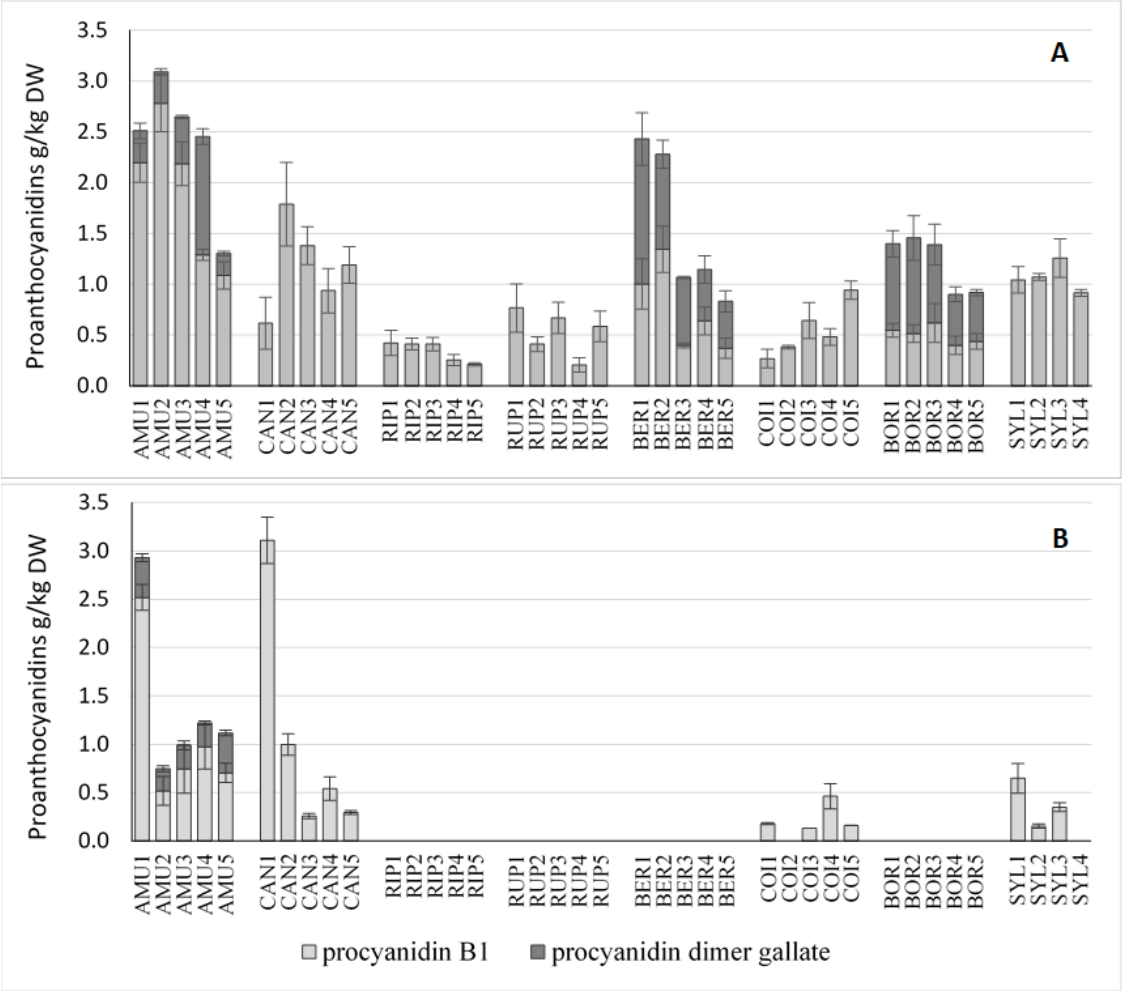

Figure 7. Accumulation of proanthocyanidin in *Vitis sp*. leaves (A = blades; B = veins) during the vegetative season. Means of three replicates ± SE; see Figure 5 for genotype acronym specification.

3.1.5 Flavanonols.

In blades, flavanonols were detected exclusively in *V. amurensis* and *V. coignetiae*, whereas in veins, they were also found in *V. v. sylvestris*. (Figure 8; Supplementary Table 4A, B). In blades, concentration was much lower than in veins. Particularly high concentration was found in *V. amurensis* veins with values ranging between 0.95 and 2.12 g kg^{-1} (Figure 8B). In both Asian species, vein total flavanonol concentration showed two peaks, the first at DOY 148 and the second at DOY 215 whereas in *V. v. sylvestris* no differences among sampling times were noticed. Three flavanonol glycosides were quantified and identified in leaves by the characteristic absorbance maximum at 290 nm and correspondent mass spectra (Table 3): dihydroquercetin-hexoside was the main flavanonol found in *V.*

amurensis and *V. coignetiae* where it was the exclusive flavanonol in blades and it accounted for up to 90% of total flavanonols in veins (Figure 8A,B). In addition, dihydrokaempferol-hexoside was detected in *V. amurensis* and in *V. coignetiae* veins with no differences among sampling dates. Astilbin (dihydroquercetin-rhamnoside) was exclusively detected in the Eurasian species *V. v. sylvestris* veins.

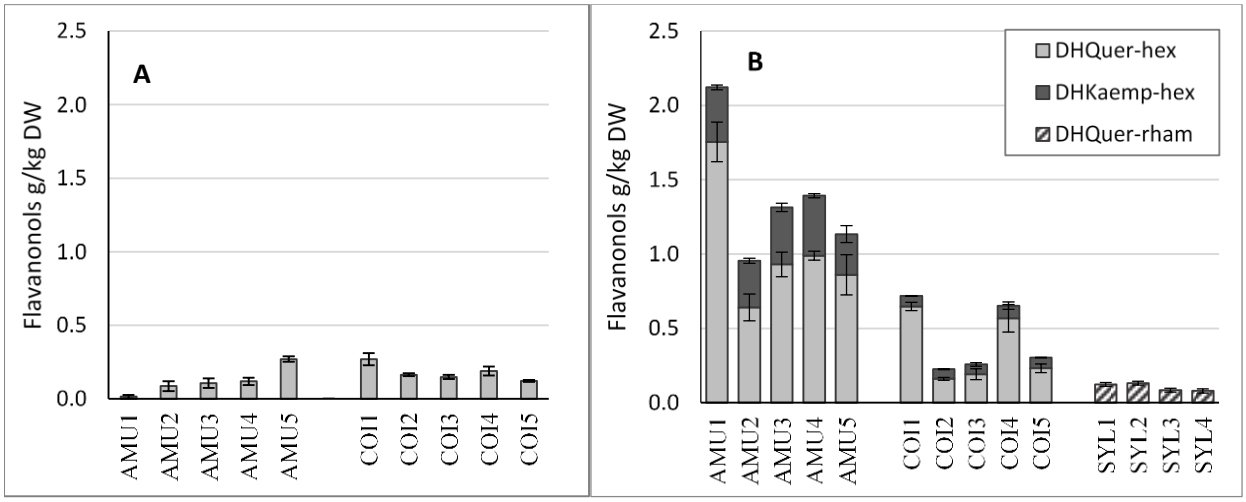

Figure 8. Accumulation of flavanonol content in *Vitis sp.* leaves (A = blades; B = veins) during the vegetative season. Means of three replicates ± SE; DHQuer - dihydroquercetin; DHKaemp – dihydrokaempferol; hex – hexoside; rham – rhamnoside; see Figure 5 for genotype acronym specification.

3.1.6 Flavones

Flavones have rarely been detected in leaves and other vegetative organs of grapevine. In fact, also in our analyzed genotypes, flavones accumulated exclusively in *V. berlandieri* and Börner. Total flavone concentration ranged between 2.5 and 4.9 g kg^{-1} DW in blades (Figure 9A). In comparison with *V. berlandieri*, Börner blades accumulated significantly higher concentration of total flavones from DOY 148 (end of May) until DOY 195 (mid-July). Similar to the other classes of measured polyphenols, flavone concentration was lower in veins respect to blades, ranging from 0.7 to 1.2 g kg^{-1} DW (Figure 9B). Five flavones accumulated in both tissues, two were apigenin glycosides and three luteolin glycosides. Isomers of apigenin glucosides were differentiated by pseudomolecular ion $[M - H]^-$ at *m/z* 431 and absorbance maximum at 270 nm and 337 nm. Moreover, the product ions of luteolin glycoside

and their intensities provided insight into individual compound structure. Apigenin 8-*C*-glucoside (isovitexin) product ions were detected at *m/z* 283, 311, 341 and apigenin 6-*C*-glucoside (vitexin) product ions were detected at *m/z* 283, 311, 341, 413 (Table 3). The absence of product ion m/z 413 and the lower intensity of product ion at *m/z* 341 $[^{0,3}X_0 - H]^-$ helped to differentiate apigenin 8-*C*-glucoside from apigenin 6-*C*-glucoside, accordingly to Kim et al. (2018). Isomers of luteolin glucoside had pseudomolecular ion $[M - H]^-$ at *m/z* 447 and absorbance maximum at 269, 350 nm. Furthermore, comparison of product ion pattern of three luteolin glucoside isomers let us tentatively identify luteolin 8-*C*-glucoside (isoorientin), luteolin 6-*C*-glucoside (orientin) and luteolin 7-*O*-glucoside (luteolin) (Table 3). The compound with absence of product ion at *m/z* 429 and high intensity of product ion at *m/z* 357 $[^{0,3}X_0 - H]^-$ was tentatively identified as luteolin 6-*C*-glucoside. Luteolin glucoside isomer with product ion pattern of two high abundance fragment at m/z 285 $[M - H - 162]^-$ and m/z 284 $[M - H - 162]^{-\bullet}$ was tentatively identified as luteolin 7-*O*-glucoside, according to Li et al. (2016). Leaf flavone profile analysis showed that isoorientin together with isovitexin comprised up to 70% of all detected flavones, thus 8-*C* was the predominant glycosylation site of flavone aglycones in *Vitis sp*. leaves (Figure 9; Supplementary Table 5).

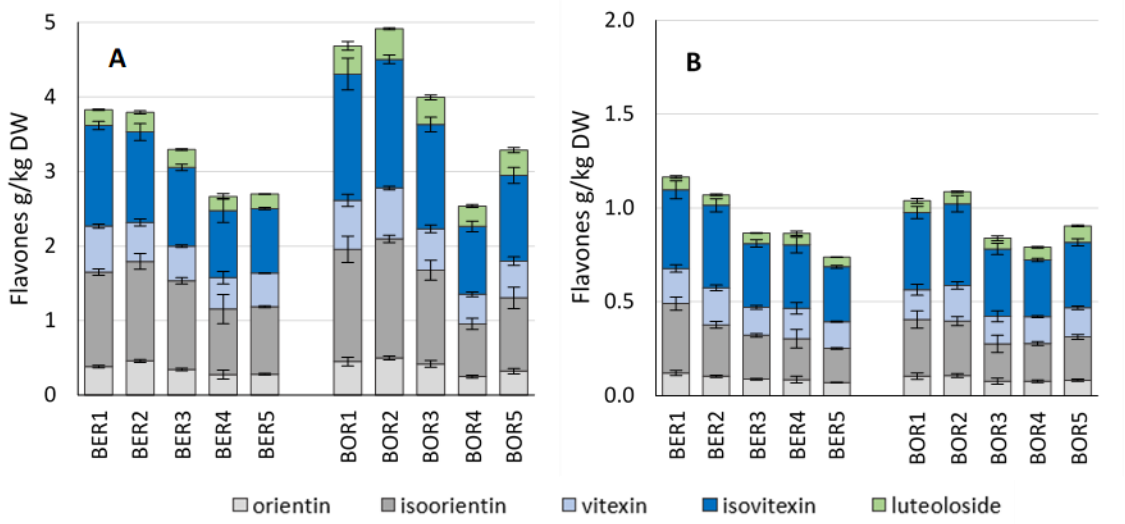

Figure 9. Accumulation of flavones in *Vitis sp.* leaves (A = blades; B = veins) during the vegetative season. Means of three replicates ± SE; see Figure 5 for genotype acronym specification.

3.2 Overall polyphenolic patterns and genotype associations

In blades, the PCA model that explained the highest variance with three PRINs (72 %), allowing a net separation of genotypes, based on eleven variables (Table 4) that were gradually chosen to exclude redundancy. Variables associated to the first PRIN were phenolic acids (the sum of fertaric acid, coutaric acid isomer and the *cis* and *trans* forms of caftaric acid) and the total concentration of dimeric proanthocyanidins. Genotypes separated on the second PRIN on the basis of myricetin glycoside (glucoside + glucuronide) concentration and of flavonol total amount. The third PRIN separated genotypes based on total flavan-3-ol concentration, evidencing in particular, *V. amurensis*. Considering that the eigenvalue on PRIN3 was almost 2.0 (1.79, Table 4), the flavone total concentration could be considered as the second variable associated (negatively) to the third PRIN. On this basis, Börner and *V. berlandieri*, highly associated to the third PRIN negative values, evidenced their peculiar capability to accumulate high amounts of flavones in leaf blades (Figure 9; Figure 10).

In veins, the proposed PCA model (Table 5; Figure 11) explained the 75 % of total variance with the first three PRINs. Variables associated to the first PRIN were total polyphenols, total flavan-3-ols and flavonol glucuronic derivatives; these variables did not markedly identify varieties or specific time-points of the leaf vegetative cycle, with the only exception of *V. amurensis,* generally associated to positive values of the first PRIN. Flavanonols and glucosides of flavonols were negatively associated to the second PRIN. Dimeric proanthocyanidins associated to the third PRIN, allowed to evidence that in genotypes accumulating this class of compounds, concentration was higher at the first sampling (particularly in *V. amurensis*, *V. candicans*, *V. berlandieri* and in Börner).

3.3 Seasonal polyphenol-related traits

The flavonol concentration varied during the vegetative season, but the profile, strictly related to a specific genotype, did not change (Figure 4).

The phenolic acid profile was species-specific, as well, with *V. rupestris* showing the highest diversification both in blades and in veins. *Trans*-caftaric acid (the main phenolic acid) percentage incidence was generally stable during the season or it decreased in a few genotypes (Figure 5).

The proanthocyanidin profile evolved during the season in a genotype-specific manner in blades; oppositely, it did not change in veins (Figure 7).

No major differences were noticed in relation to the flavone profile evolution in *Vitis berlandieri* and in Börner during the season.

Table 4. Eigenvectors of eleven variables (polyphenols in leaf blades) on the first three principal components (PRIN1, PRIN2 and PRIN3).

	PRIN1	**PRIN2**	**PRIN3**
Myricetin glycosides (glucoside + glucuronide)	-0.25	**0.51**	-0.05
Protocatechuic acid-glucoside	-0.24	-0.04	0.20
Dihydroquercetin-hexoside	-0.14	-0.32	0.36
Fertaric acid + coutaric acid isomer	**-0.40**	0.34	-0.02
Caftaric acids (cis + trans)	**0.41**	0.33	-014
Coutaric acids (cis + trans)	0.30	0.02	-0.02
Flavones	0.26	-0.03	**-0.43**
Total phenolic acids	0.33	0.44	-0.15
Total flavonols	-0.14	**0.46**	0.43
Total flavan-3-ols	0.30	0.08	**0.55**
Dimeric proanthocyanidins	**0.43**	0.03	0.34
Eigenvalues	3.56	2.57	1.79
Total variance	0.32	0.23	0.16

Eigenvalues of the three PRINs and their contribution to total variance. In bold letters, the variables associated to the appropriate PRIN.

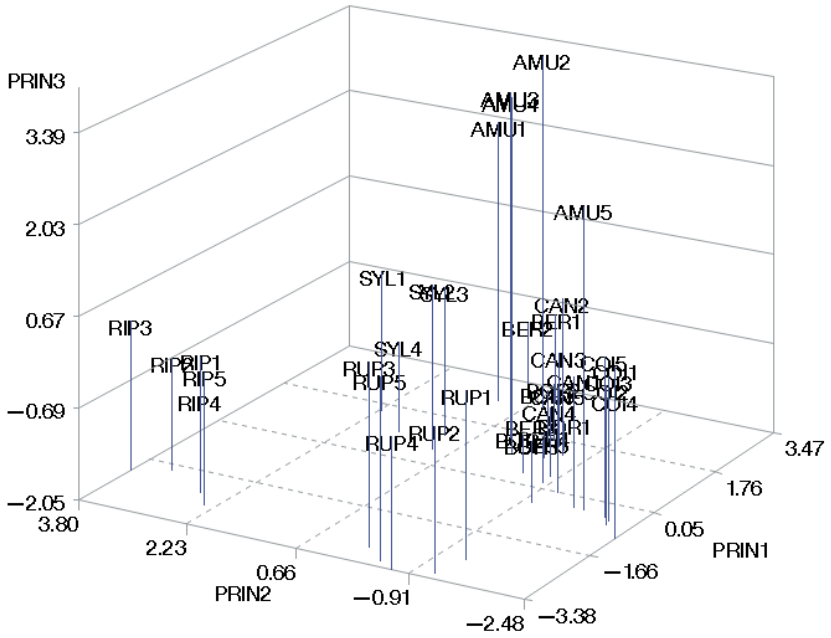

Figure 10. Tridimensional distribution of *Vitis* species during the vegetative season (1 to 5) on the first three principal components, obtained by polyphenol quantifications and profiles of leaf blade extracts (see Table 4 for the list of used variables).

Table 5. Eigenvectors of nine variables (polyphenols in leaf veins) on the first three principal components (PRIN1, PRIN2 and PRIN3).

	PRIN1	**PRIN2**	**PRIN3**
Total polyphenols	**0.50**	0.11	-0.14
Total phenolic acids	0.36	**0.39**	0.25
Total flavan-3-ols	**0.38**	0.32	0.38
Dimeric proanthocyanidins	0.30	-0.30	**0.52**
Total flavanonols	0.18	**-0.56**	0.21
Total flavones	-0.22	0.35	0.28
Sum of glucuronide flavonols	**0.38**	0.14	-0.33
Sum of glucoside flavonols	0.36	-0.38	-0.29
Sum of rhamnoside flavonols	0.15	0.21	-0.42
Eigenvalues	3.27	2.17	1.34
Total variance	0.36	0.24	0.15

Eigenvalues of the three PRINs and their contribution to total variance. In bold letters, the variables associated to the appropriate PRIN.

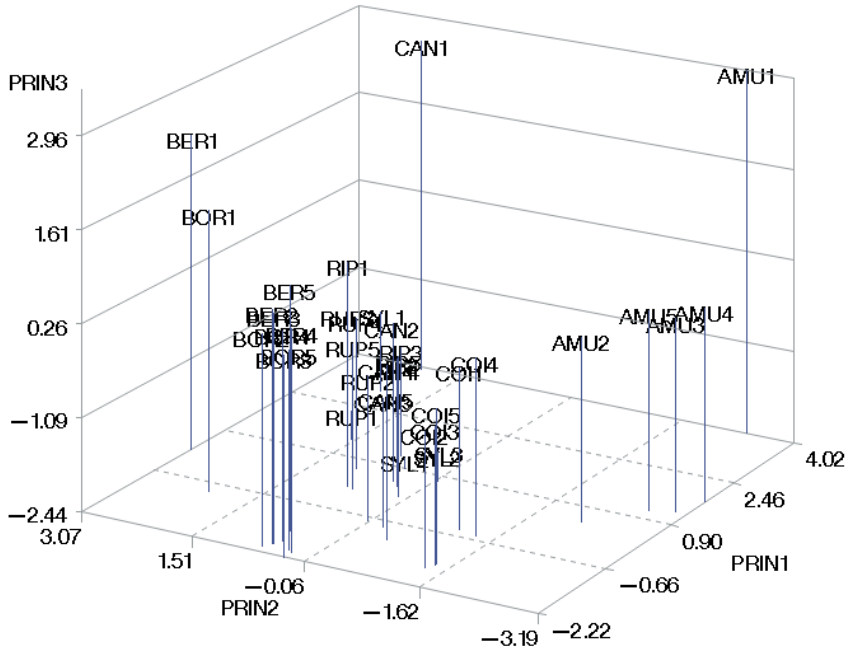

Figure 11. Tridimensional distribution of *Vitis* species during the vegetative season (1 to 5) on the first three principal components, obtained by polyphenol quantifications and profiles of leaf vein extracts (see Table 5 for the list of variables used).

4. DISCUSSION

There is increasing interest in grapevine leaf polyphenols to deepen knowledge about their biological role in plant-defense mechanisms and to answer to the growing demand of natural bioactive compounds for the feed/food, pharmaceutical and cosmetic sectors. Grapevine leaves are already employed in the production of food ingredients, dietary supplements, pharmaceutical products and medicated cosmetics (Dani et al., 2010). The evolution of the polyphenolic composition of eight *Vitis* genotypes during the vegetative season allowed pointing out significant qualitative and quantitative differences.

4.1 Genotype-related specific traits of leaf polyphenols

The two studied American species, *V. riparia* and *V. rupestris*, belonging to the same subgeneric group of *Ripariae* (Table 1), involved in crossbreed of Regent, a cultivar displaying low susceptibility to *Plasmopara viticola* and to *Erysiphe necator*, shared common phenolic composition and accumulation trends. In particular, they showed similarities in the accumulation trend of total phenolic

acids and total flavan-3-ols. Additionally, they displayed identical flavonol profiles (Figure 4). However, *V. riparia* accumulated higher concentration of total polyphenols, total flavonols and total phenolic acids respect to *V. rupestris*, which could justify its specifically high resistance to downy mildew, as both flavonols and phenolic acids have been associated with the ability to limit this pathogen diffusion (Ali et al., 2012). Moreover, *V. riparia* total flavonol concentration both in blades and in veins was the highest comparing to all the other analyzed genotypes, almost during the entire vegetative season (Figure 3).

The American genotypes Börner and *V. berlandieri*, known as low-susceptible to many diseases, were classified by Galet (1988) into the same subgeneric serie of *Cinereae* (Table 1). Their main leaf polyphenolic traits showed many similarities, such as the concentration and the accumulation trend of total polyphenols, total flavonols and total flavones, together with identical profiles of flavonols, proanthocyanidins and flavones. Therefore, Galet's classification based on plant morphology matched with phenol profile patterns of these two genotypes, suggesting a phylogenetic link between *Vitis cinerea* (Börner parent) and *Vitis berlandieri*. Flavones, bioactive compounds often extracted from medical and herbaceous plants and involved in plant signaling and defense responses (Jiang et al., 2016), were exclusively detected in Börner and in *V. berlandieri*, among the studied genotypes. In grapevine, they are generally considered as minor compounds mostly represented by luteolin, but in Börner and *V. berlandieri* leaves their concentration reached 5 g kg^{-1} DW (Figure 9) and showed a much wider profile, consisting of five apigenin and luteolin derivatives: specifically, four of them were *C*-glycosides and one *O*-glycoside (Table 3; Figure 9). *C*-glycoside flavonoids generally display higher antioxidant potential and better therapeutic properties compared to aglycons or to *O*-glycosylated flavonoids (Xiao et al., 2016). However, as most studies focused on biological activities of the more common plant *O*-glycosylated-flavonoids, further research is required on grapevine *C*-glycosylated derivative health beneficial effect. McNally et al. (2003) demonstrated that in cucumber *C*-flavones acted as phytoalexins in response to powdery mildew fungus *Podosphaera xanthii* and concentration increases of orientin and isoorientin were reported to be induced by soil salinity in buckwheat (*F. esculentum*) (Yang et al., 2018).

512 *V. berlandieri* and Börner were also similar in the fact that they did not accumulate kaempferol
513 glycosides, in line with what was previously demonstrated in the grapevine series *Cinerea* (Moore and
514 Giannasi, 1987).

515 The Asian wild species *V. coignetiae* has been cultivated during the last two decades in Japan
516 for wine, juice and jam making (Kamiya et al., 2018). *V. coignetiae* leaf extract showed a strong radical-
517 scavenging activity and potential hepato-protective effect on nonalcoholic steatohepatitis in liver
518 (Takayama et al., 2009). However, in *V. coignetiae* leaves of the present study, the concentration of leaf
519 polyphenols was generally lower comparing to the other genotypes (Figure 2). *V. coignetiae* along with
520 *V. amurensis* and *V. v. sylvestris* was one of the few species which accumulated flavanonols; two
521 flavanonol compounds were detected in *V. coignetiae*, dihydrokaempferol-hexoside in veins and
522 dihydroquercetin-hexoside in both blades and veins (Figure 8A, B). Taxifolin (dihydroquercetin)
523 glycoside is known to have anti-inflammatory activity (Kim et al., 2008) and potential positive effect
524 on atopic dermatitis treatment (Ahn et al., 2010).

525 The most peculiar species among the analyzed genotypes was *V. amurensis*, which is known for
526 its cold tolerance and disease resistance to downy mildew (Jürges et al., 2009), anthracnose and white
527 rot (Li et al., 2008). The leaves of *V. amurensis* reached the highest concentration of total polyphenols
528 and flavan-3-ols in blades, important amounts of flavonols, and the highest concentration of flavanonols
529 in veins. *V. amurensis* leaves are used in conventional Chinese medicine and included in Korean herbal
530 Pharmacopoeia (Chen et al., 2018). Moreover, leaf extracts showed antimicrobial activity against
531 *Streptococcus mutans* and *Streptococcus sanguis* (Yim et al., 2010) and neuroprotective effect (Jeong
532 et al., 2010). Bak et al. (2012, 2016) demonstrated that *V. amurensis* seed proanthocyanidins have
533 hepato-protective and antioxidative properties and a possible chemopreventive role in humans
534 hepatocarcinoma cells. In *V. amurensis* blades, concentration of total flavan-3-ols was significantly
535 higher during the entire vegetative season comparing to the other genotypes (Figure 6A). Moreover, also
536 the concentration of (-)-epicatechin was particularly high. This compound was previously individuated
537 as a reaction of pear leaves against *Erwinia amylovora* and of apple leaves against *Venturia inaequalis*

(as discussed in Kedrina-Okutan et al., 2018). In non-*Vitis vinifera* genotypes, (-)-epigallocatechin was additionally detected with increasing concentration during the vegetative season (Figure 6). (Epi)gallocatechin-(epi)catechin was tentatively identified in *V. amurensis*, consistently with previously published dimeric PA identification (Flamini, 2013). (Vagiri et al., 2017) reported a negative correlation between (-)-epigallocatechin and the presence of septoria leaf spot caused by *Mycosphaerella ribis* in black currant leaves. The high concentration of constitutive polyphenolic compounds throughout the season in *V. amurensis* leaves contributes to explain this biotype excellent ability to protect against adverse environmental condition (frost and diseases). Furthermore, the abundance of polyphenols in *V. amurensis* leaves emphasizes that this species has the potential for further use as a source of natural bioactive compounds for nutraceutical and pharmacological uses.

The polyphenolic composition of *V. candicans* leaves was studied here for the first time, to the best of our knowledge. This American species has been described as highly vigorous, highly resistant to downy mildew and tolerant to drought and salt (Ollat et al., 2016). *V. candicans* leaves displayed a low concentration of total polyphenols, total flavonols, but a relatively high concentration of proanthocyanidins and this genotype was among the few able to accumulate procyanidin B_1 in veins (Figure 7). Procyanidin B_1, the dimeric proanthocyanidin consisting in units of (-)-epicatechin and (+)-catechin, has an ecological significance in protecting plants against pathogens, insect pests and larger herbivores (Dixon et al., 2005). For instance, previous studies demonstrated that *B. cinerea* remained quiescent in immature strawberry until B proanthocyanidins are present at an essential high concentration: this effect is related to the proanthocyanidin inhibitory potential of the fungal polygalacturonase (Jersch et al., 1989).

The Eurasian wild species *V. v. sylvestris*, the ancestor of the worldwide cultivated *V. vinifera sativa*, accumulated relatively high concentration of total polyphenols and total phenolic acids (Figure 2; Figure 5). Among phenolic acids, the predominant non-flavonoid polyphenols, caftaric acid was the prevalent form, representing up to 90% of total phenolic acids (Figure 5C), similarly to what was previously assessed in *V. vinifera sativa* leaves (Kedrina-Okutan et al., 2018). Caftaric acid is an

564 important bioactive component found in chicory, in the medical plant *Echinacea purpurea* (Bel-Rhlid
565 et al., 2012) and, in *V. coignetiae* juice, where it exhibited anti-mutagenic and anti-inflammatory
566 properties (Kamiya et al., 2018). In *V. v. sylvestris* leaves, we found astilbin (Figure 8B), in
567 concentrations that were in line with those found in Cabernet Sauvignon, Grenache, Shiraz and Barbera,
568 that were much lower respect to those found in Nebbiolo and Pinot noir (Kedrina-Okutan et al., 2018).
569 Additionally, *V. v. sylvestris* displayed the most complex flavonol profile (Figure 4). In *V. v. sylvestris*
570 veins we identified and quantified seven flavonols out of the eight flavonol compounds
571 chromatographically separated; *V. v. sylvestris* was the exclusive genotype that accumulated kaempferol
572 3-*O*-rhamnoside (Figure 4, Table 3) and, compared to *V. vinifera sativa*, it accumulated additional
573 flavonol rhamnosides (kaempferol- and quercetin-3-O-rhamnoside), showing a wider flavonol-profile
574 diversification.

575 The peculiar traits of individual *Vitis* species leaf polyphenolic compositions were highlighted
576 by the proposed PCA models. In particular, *V. riparia* and *V. rupestris* displayed similar eigenvectors
577 on PRIN1 and PRIN3, highlighting their similar concentrations of dimeric proanthocyanidins, similar
578 percentage incidence of caftaric acid over total phenolic acids (on PRIN1) and similar concentration of
579 flavan-3-ols on PRIN3 (Table 4; Figure 10). *V. riparia* sharply distinguished on PRIN2 due its high
580 flavonol content (Figure 3A) and the highest percentage incidence of myricetin derivatives respect to
581 the other analyzed genotypes (Figure 4). Flavonols allowed the species separation, also when
582 considering their concentration: in fact, flavonol glucosides, together with flavanonols, allowed a sharp
583 separation of *V. amurensis* samples and were negatively associated to the second PRIN of the vein PCA
584 model (Figure 11; Table 5). Oppositely, *V. berlandieri* and Börner associated to positive values of
585 PRIN2, with reduced amounts of flavonol-glucosides comparing to the other species. Quercetin 3-O-
586 glucoronide was the prevalent flavonol in the analyzed genotypes, consistently with other studies on *V.*
587 *vinifera* and *V. labrusca* leaves (Dresch et al., 2014; Kedrina-Okutan et al., 2018). The differences in
588 the flavonol profile might be related to the genus *Vitis* evolution: in fact, evidences about the reduction
589 in structural complexity and in molecule diversification were reported (Moore and Giannasi, 1994), and

590 they were also ascribed to the flavonoid biosynthetic pathway, that was shown to have undergone a
591 simplification during evolution, particularly in domesticated genotypes. Interestingly, *V. v. sylvestris*,
592 which, being the wild form of *V. v. sativa*, underwent no or low selection pressure, showed the widest
593 flavonol profile diversification and comprised high concentration of quercetin 3-*O*-rhamnoside (22% of
594 total flavonols); besides, *V v. sylvestris* also displayed a higher flavonol profile diversity respect to
595 cultivated varieties of *V. vinifera*. Quercetin 3-*O*-rhamnoside was also found in *V. berlandieri* and in
596 Börner. Hilbert et al. (2015) identified flavonol rhamnosides in berry skin extracts of *V. cinerea*, which
597 is native to the United States and belongs to the subgeneric group of *Cinereae* (Galet, 1988), the same
598 of *V. berlandieri* and Börner. Thus, flavonol rhamnosides seem to be genotype-specific compounds,
599 therefore they have the potential to be exploited as specific markers of some species and/or subspecies.
600 Vein flavanonol concentration, associated to PRIN2 (Figure 11; Table 5) allowed the sharp
601 separation of *V. amurensis*, and, secondarily the separation of *V. coignetiae* and *V. v. sylvestris*. Since
602 flavanonols accumulate prevalently in leaf veins, their concentration on a per whole leaf basis is
603 supposed to be low, considering the limited percentage incidence of veins on the total leaf area, but the
604 role of these tissue-specific compounds arouses the interest about their interaction with vein-located
605 pathogens. In some *V. vinifera* s*ativa* varieties, we previously identified these molecules, particularly in
606 veins (Kedrina-Okutan et al., 2018) where astilbin was the predominant compound. We also associated
607 astilbin accumulation to a possible defense-mechanism in cv Nebbiolo (Ferrandino et al., 2019). In fact,
608 in Nebbiolo leaves of plants affected by *Flavescence dorée* (FD), we found a significantly higher
609 concentration of flavanonols compared to healthy vines, hypothesizing a possible capability to limit the
610 pathogen development. Interestingly, together with *V. v. sylvestris*, also *V. amurensis*, whose capability
611 to defend against pathogens is detailed and *V. coignetiae*, known for its strong antiradical role,
612 accumulate flavanonols.
613 As to the ecological role of leaf polyphenols, further studies would be of extreme interest to
614 understand: i) if genotype-related molecules such as flavanonols and flavones have specific capacities

615 to limit diseases, starting from *in vitro* trials, ii) if grapevine plants can be induced to produce specific
616 molecules, once demonstrated that they can efficaciously serve to limit pathogen development.
617 As to the nutraceutical, pharmaceutical and feed/food ingredients, grapevine leaf are a source of
618 bioactive compounds that in future could be economically exploited, also exploiting their wide and still
619 little investigated, biodiversity.
620
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