PAPER

GRAPEVINE CANES WASTE FROM VENETO REGION AS A NEW SOURCE OF STILBENOIDS CONTENT

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ABSTRACT

In the present paper we analyzed the stilbene accumulation in grape canes of seven autochthonous grape varieties from Veneto region, Italy, in comparison to two international cvs. In addition, we investigated the effect of pruning time on the stilbenes accumulation rate during the storage. Taking into account the effect of both pruning time (October, November and December) and storage time (from zero to twelve weeks at room temperature), cultivar Verdiso and Incrocio Manzoni 13.0.25 showed the highest accumulation of *trans*-resveratrol, *trans*-piceatannol, and *trans*-ε-viniferin, in particular when the canes were harvested in October, highlighting the importance of the cultivar but also the effect of the pruning time on the accumulation of stilbenes in grape canes.

Keywords: autochthonous varieties, grape canes, piceatannol, resveratrol, viniferin

1. INTRODUCTION

The winemaking industry is responsible for large part of grape waste as pomace, grape canes, seeds and stems. Some waste as pomace and seeds are valued from food industry being popular as source of antioxidant polyphenols or for grape seed oil production. At the same time, there is still a need for alternative sources of resveratrol, as can be seen by the recent permission of resveratrol as a novel food ingredient in the European Union (Commission Implementing Decision (EU) 2016/1190). Stilbenoids are a small family of plant secondary metabolites derived from the phenylpropanoid pathway. They act as plant phytoalexins displaying different bioactivities and thus making them compounds of high current interest (FERNANDEZ-MAR *et al*., 2012). In the Vitaceae, stilbenoids accumulate in response to various biotic and abiotic stresses such as the attack of pathogen *Erysiphe necator, Plasmopara viticola, Botrytis cinerea* and UV-C irradiation (SCHNEE *et al*., 2008; SCHNEE *et al*., 2013; PEZET *et al*., 2004; ALONSO-VILLAVERDE *et al*., 2011; ADRIAN and JEANDET, 2012; GRUAU *et al*., 2015; YIN *et al*., 2016). They can also be induced in response to plant hormones, such as ethylene and jasmonate (D'ONOFRIO *et al*. 2009; JIANG *et al*., 2015). In grapevine, the stilbene *tran*s-resveratrol has attracted particular attention, not only because of its antimicrobial activity, but also due to its health benefits to humans, as antioxidant, anticarcinogenic, anti-inflammatory, cardioprotective and neuroprotective, among others (FLAMINI *et al*., 2013; BAVARESCO *et al*., 2012; SHEN *et al*., 2009).

Stilbenoids accumulate in different parts of grapevine, however, WANG *et al.* (2010) found the highest concentration of *trans*-resveratrol in grape canes. Grape canes waste is generated during winter annual pruning and represents a large source of waste derived from the viticulture industry, with an estimated volume of 1 to 3 t/ha year depending upon plantation density, climate, and vigor of the grape variety (DEVESA-REY *et al*., 2011; EWALD *et al*., 2017). Currently, emission protection regulations mostly prohibit the burning of grape canes, which was the traditional way of disposal of these woody residues (EWALD *et al*., 2017).

Grape canes can be considered as an unexploited source of stilbenoids, as proposed by several authors (VERGARA *et al*., 2012; LAMBERT *et al*., 2013; GORENA *et al*., 2014; HOUILLÉ *et al*., 2015; GUERRERO *et al*., 2016; EWALD *et al*., 2017). Different content of stilbenoids have been found in grape canes of *Vitis vinifera* stored at 40 to 45°C or at room temperature (20±3°C). VERGARA *et al.* (2012) compared the stilbenoid content in canes of several grape varieties cultivated in different regions and in two different years in Chile, finding *trans*-resveratrol in the range 446 to 6533 mg/kg DW, with the highest content found in Gewurztraminer variety. Lower values of *trans*-resveratrol were reported by LAMBERT *et al.* (2013) comparing grape canes harvested from 16 different varieties in France. These authors found the lowest content of *trans*-resveratrol in Chardonnay (190 mg/kg DW) and the highest content in Pinot noir cultivar (1526 mg/kg DW) while the *trans*-piceatannol and *trans*-ε-viniferin content were significantly higher in all cultivars when compared with VERGARA *et al.* (2012). GUERRERO *et al.* (2016) described that most abundant stilbenoid was *trans*-viniferin in all cultivars, which reached the highest concentration in Gewürztraminer cultivar. While EWALD *et al.* (2017) found the higher levels of *trans*-resveratrol and *trans*-viniferin in Pinot blanc and Sauvignon blanc harvested in Germany (3199-3329 mg/kg DW, respectively). ZHANG *et al.* (2011) studied the content of *trans*-resveratrol in grape canes of many different grape varieties, including local varieties cultivated in the seven major Chinese grape producing regions finding high variability.

Beside the genetic determinants, several other factors could explain these different results, such as the climate, the solvent used for the extraction of stilbenoids (RAYNE *et al.*, 2008), the temperature and time of grape canes storage (HOUILLÉ *et al.*, 2015). In addition, other factors, such as the pruning time, could affect the stilbene accumulation rate in canes. This factor has never taken in account before, in fact in many articles this data is not reported at all, and, when present, show to be highly variable, with pruning times varying from 1 to 4 months after the grape harvest.
Up to date there are no data available concerning stilbenoid content in grape cane waste of Italian grape varieties. In the present paper the stilbene accumulation in grape canes of seven autochthonous grape varieties from Veneto region, one of the most important wine producing regions in Italy, has been studied. In addition, the effect of pruning time on the stilbenes accumulation rate during the storage was taken in account.

2. MATERIALS AND METHODS

2.1. Plant materials

Grape canes of *Vitis vinifera* L. from Veneto region white varieties, such as Bianchetta, Glera VCR Sel. Lungo, Incrocio Manzoni 6.0.1.3, Verdiso; and red varieties Incrocio Manzoni 13.0.25, Marzemino Biotipo 13, Raboso, were collected randomly from plants from a conventional vineyard at Oenological School of Conegliano, Province of Treviso, Italy (I.S.I.S.S – Istituto Statale di Istruzione Secondaria Superiore "G.B. Cerletti") (latitude 45° 87′ 69″ N and longitude 12° 28′ 53″ E). As reference, international varieties Sauvignon blanc INRA 316 and Pinot noir, grown in the same vineyard, were chosen because according to LAMBERT *et al*. (2013) these were the cultivars with the highest content of stilbenes. The canes were collected monthly in October (11^{th}), November (15^{th}) and December (13^{th}) (autumn-winter 2016-2017) from 30 selected plants for each variety. About 1.5 kg for each sampling and for each variety were obtained. The canes were cut into 10-20 cm long pieces and stored for three, six, nine and twelve weeks in well-aerated conditions in the dark, at room temperature. For control, a sample was immediately extracted after each pruning sampling point.

2.2. Stilbenoid extraction

The stilbenoid extraction was performed according to the procedure described by RAYNE *et al.* (2008) with some modifications. Briefly, the grape canes were ground with a coffee grinder (Imetec, Azzano San Paolo, BG, Italy). Three-stage extraction (in the dark to avoid stilbene isomerization) was performed by continuous stirring at room temperature using an 8:1 (v/w) 80% ethanol:sample ratio over a 60-min period for each extraction. During the first extraction, 250 μL of t-OH-stilbene 200 μg/mL in ethanol were added as internal standard. The extracts were vacuum filtered at 1.6 µm on glass microfibre filter (GF/A, Whatman) and combined and the solvent removed by rotary evaporation (Büchi model R-114, Flawil, Switzerland), then stored at -20°C. All the extractions were performed in triplicate. Before quantification, the extracts were defrosted at room temperature and homogenized. An aliquot of the extract (500 μL) was transferred to Eppendorf tubes and 500 μL of methanol were added. After centrifugation at 4000 × g for 1 min, part of the supernatant (500 μL) was transferred to HPLC vials.

2.3. HPLC analysis

The analysis of stilbenoids was performed according to the procedure described by VINCENZI *et al.* (2013) with some modifications. Stilbenes were separated on a C18 Lichrospher column (4 mm x 250 mm, 5 μm, Agilent Technologies, Milano, Italy) at 40°C, using an HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Dual Band UV detector Waters 2487 (Waters Corporation, Milford, MA, USA). The mobile phase gradient was 0.5% v/v formic acid in deionized water (solvent A) and 2% v/v formic acid in methanol (solvent B).

The gradient program was 0 to 10% (solvent B) in 3 min, followed by 10 to 30% (solvent B) in 5 min, 30 to 44% (solvent B) in 35 min, 44 to 55% (solvent B) in 2 min, 55 to 75% (solvent B) in 15 min and 75 to 100% (solvent B) in 1 min. After washing for 2 min with solvent B, the column was re-equilibrated with solvent A. The flow rate was 1.0 mL/min and injection volume 20 μL. Detection was performed at 306 nm for *trans*-isomers for *trans*-resveratrol, *trans*-piceatannol and *trans*-ε-viniferin and at 285 nm for the corresponding *cis*-isomers. All the stilbene standards were obtained in *trans* form from Extrasynthese (Genay Cedex, France). The *cis*-isomers were obtained by UV-exposition of the corresponding *trans*-isomers, and were loaded in HPLC for the identification of the retention times. The concentration of individual stilbenes (both *trans*- and *cis*-forms) was calculated on the basis of peak areas using calibration curves of commercially available standards of *trans*-resveratrol, *trans*-piceatannol and *trans*-ε-viniferin, and correcting the value for the internal standard recovery. Data were analysed by the Waters BreezeTM Chromatography Software (Version 3.30). The limits of detection (LOD) and quantification (LOQ) were performed according to the procedure described by (SHRIVASTAVA and GUPTA, 2011).

2.4. Statistical analysis

Within each factor the results were evaluated by one-way analysis of variance (ANOVA), and values were analyzed by Tukey's test using the software STATISTICA 12.0 (StatSoft Inc, Tolson, USA). Results were expressed as mean values ± standard deviation (SD) and the value of $p < 0.05$ was considered statistically significant.

For the global analysis of all data in order to take in account the interactions among different variables, a MANOVA test was applied and values were analyzed by Wilks's test using the software XLSTAT (Addinsoft).

3. RESULTS AND DISCUSSION

Considering that stilbenoid accumulation in cut canes depends on activation of related genes followed by active synthesis of resveratrol and its derivatives, as already reported by HOUILLÉ *et al.* (2015) and BILLET *et al.* (2018), it is expected that different grape varieties respond in different way after the injury for both total amount of stilbenoid produced and rate of their accumulation. Also, the climate and other environmental factors can affect the way the canes respond during the storage period, for this reason the canes of the seven varieties taken in consideration in this study were collected in the same year from plants grown in the same vineyard.

The evolutions of stilbenoids during the storage time for samples of different varieties harvested at different times is reported in Figs. 1, 2 and 3.

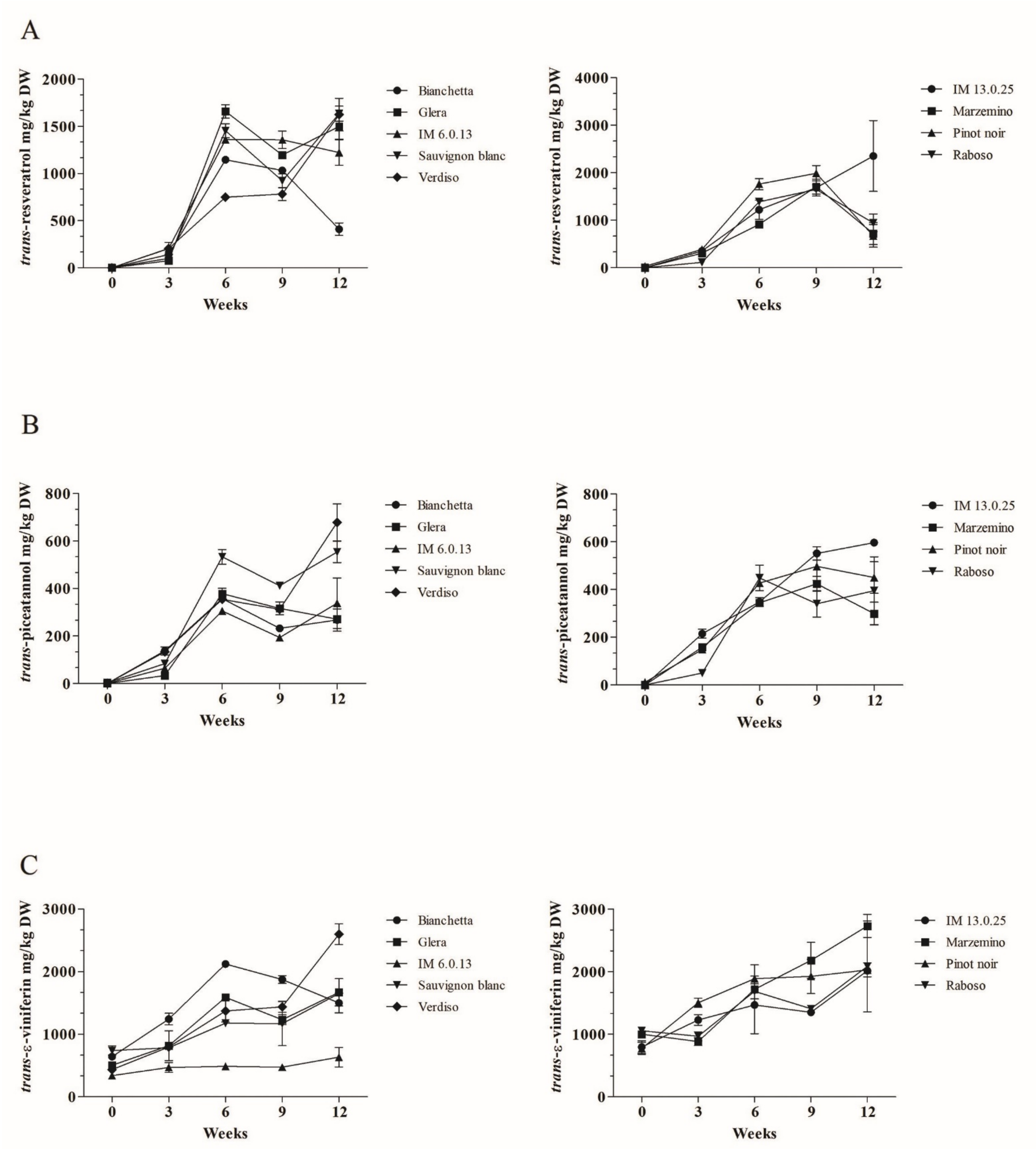

Figure 1. Content of *trans*-resveratrol (A), *trans*-piceatannol (B) and *trans*-ε-viniferin (C) on grape canes harvested in October (autumn−winter 2016−2017) from white varieties, Bianchetta, Glera, Incrocio Manzoni 6.0.1.3, Sauvignon blanc, Verdiso, and red varieties Incrocio Manzoni 13.0.25, Marzemino, Pinot noir, Raboso, and stored at room temperature for three, six, nine, and twelve weeks. Results represent the mean ± SD of triplicate assays.

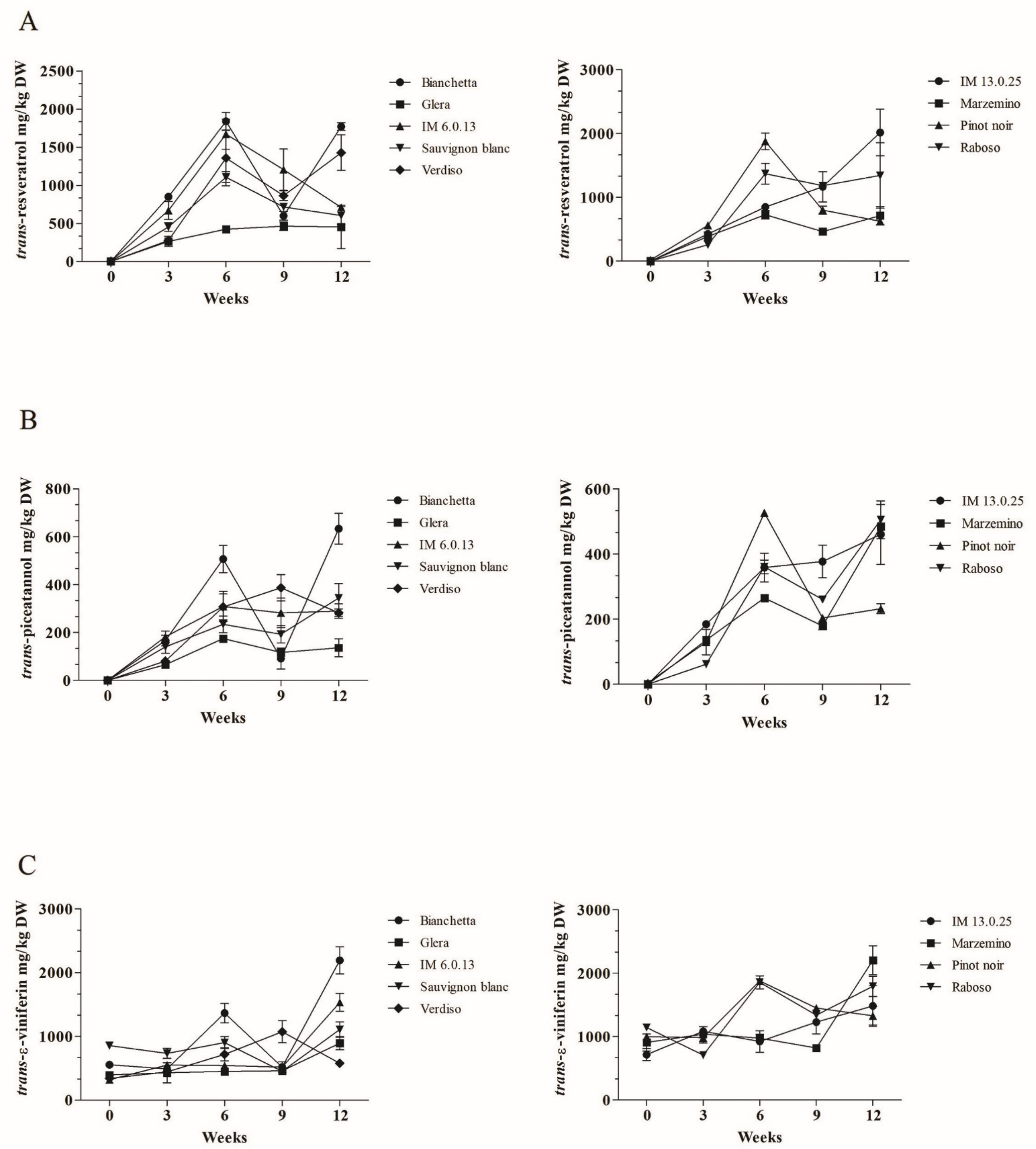

Figure 2. Content of *trans*-resveratrol (A), *trans*-piceatannol (B) and *trans*-ε-viniferin (C) on grape canes harvested at pruning time in November (autumn−winter 2016−2017) from white varieties, Bianchetta, Glera, Incrocio Manzoni 6.0.1.3, Sauvignon blanc, Verdiso, and red varieties Incrocio Manzoni 13.0.25, Marzemino, Pinot noir, Raboso, and stored at room temperature for three, six, nine, and twelve weeks. Results represent the mean ± SD of triplicate assays.

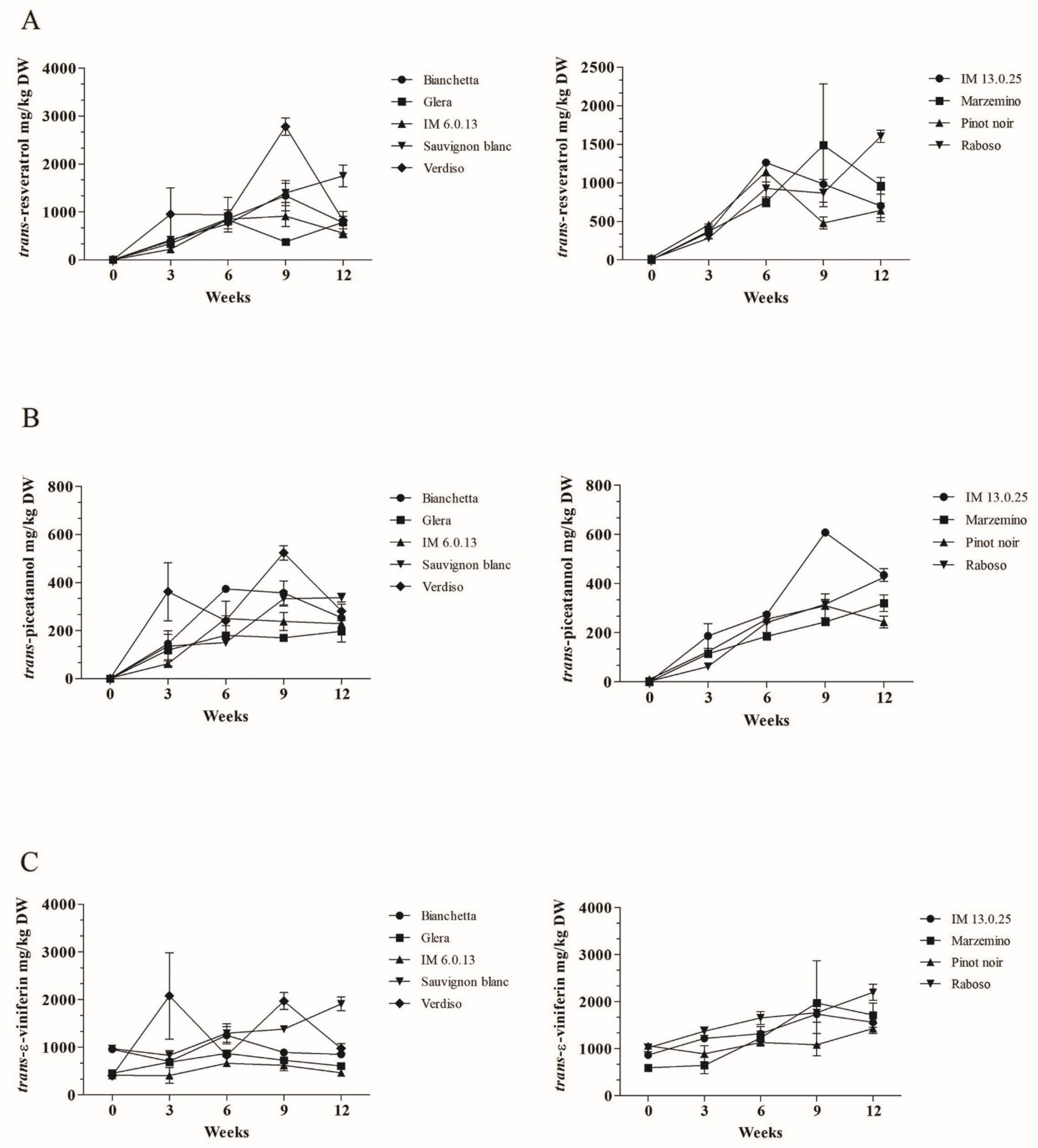

Figure 3. Content of *trans*-resveratrol (A), *trans*-piceatannol (B) and *trans*-ε-viniferin (C) on grape canes harvested at pruning time in December (autumn−winter 2016−2017) from white varieties, Bianchetta, Glera, Incrocio Manzoni 6.0.1.3, Sauvignon blanc, Verdiso, and red varieties Incrocio Manzoni 13.0.25, Marzemino, Pinot noir, Raboso, and stored at room temperature for three, six, nine, and twelve weeks. Results represent the mean ± SD of triplicate assays.

A MANOVA test was also applied to study the effect of different variables on the stilbenoids accumulation (Table 1).

Table 1. Results of MANOVA analysis on the total dataset.

	Storage	Harvest	Variety	Storage* Harvest	Storage* Variety	Harvest* Variety	Storage* Harvest* Variety
F-value	49,240	5,227	4,579	6,615	4,387	2,624	2,313
GDL1	12	6	24	21	96	48	150
GDL2	421	318	462	457	477	474	478
p-value	< 0,0001	< 0,0001	< 0,0001	< 0,0001	< 0,0001	< 0,0001	< 0,0001

As a first observation, the basal level of *trans*-piceatannol and *trans*-resveratrol in fresh canes of Pinot noir was quite low (less than 40 mg/kg DW) and below the detection level in the other varieties, whereas the dimer *trans*-ε-viniferin was already present at concentrations 10 to 30 times higher in all the samples (Supplementary table 1). These results confirm the literature data (GORENA *et al.*, 2014; HOUILLÉ *et al.*, 2015; BILLET *et al.*, 2018), only VERGARA and colleagues (2012) found very high content of *trans*-resveratrol (between 2500 and 3500 mg/kg) already at time zero.
During the storage a large increase of stilbenoids was observed in all samples, confirming that the main variable driving their accumulation in grape canes is the storage time (highest F value in Table 1). However, a significant effect of the harvest time was also found (Table 1). Collectively, the canes pruned in October showed a gradual increase of stilbenes during all the storage period, whereas the canes collected in November showed a notable peak of accumulation of stilbene content after 6 weeks of storage for almost all the varieties (Figs. 1 and 2). HOUILLÉ *et al.* (2015) found the same results, i.e. a peak of accumulation of *trans*-resveratrol and *trans*-piceatannol after 6 weeks of storage, on eight cultivars (collected in December) and stored for two, four, six, eight and ten weeks. On the other hand, the pruning carried out in December demonstrated a different behavior in the accumulation of stilbenes (Fig. 3). For many varieties, in particular for Verdiso and Incrocio Manzoni 13.0.25 (IM 13.0.25) the peak of stilbene accumulation was retarded to 9 weeks of storage. This behavior of canes harvested at different times seems to show a slower stilbenoid response in canes harvested in October, probably due to the high quantity of stilbene synthase enzymes still present in the woody tissue, which under regulate the induction of the related genes after the injury. The synthesis of stilbenoids is instead more rapid with the evolution of canes toward winter dormancy until November, then the accumulation rate slowdown in canes harvested in December. This could explain the significant effect of the interaction storage*harvest time (Table 1).
The different cultivars showed different behaviors, and different responses to harvest time and storage conditions, as confirmed by the significant effect of variety, storage*variety and harvest*variety in the MANOVA test (Table 1).
Among the samples harvested in October, cultivar Verdiso, IM 13.0.25 and Marzemino showed the highest increase of *trans*-resveratrol, *trans*-piceatannol, and *trans*-ε-viniferin after twelve weeks at RT (Fig. 1). Among the white varieties, Verdiso is one of the last to be harvested. Similarly, IM 13.0.25 and Marzemino are, among red varieties, those with the later harvest. It is not clear if this common behavior could be related to the similar accumulation rate of stilbenes in pruned canes. Regarding resveratrol, even though after 9

weeks Pinot noir was the cultivar with the highest content, confirming the high stilbene metabolism of this variety, after 12 weeks the content in IM 13.0.25 canes increased again reaching the highest value among all the varieties taken in consideration. Even for piceatannol, IM 13.0.25 presented concentration usually higher than Pinot noir.
The canes harvested in November demonstrated an increase for all stilbene compounds when maintained for twelve weeks at RT. Again, the cultivar IM 13.0.25 presented a constant increase of *trans*-resveratrol content reaching, after 12 weeks of storage, a value up to 2016±365 mg/kg DW, comparable with those found in October. In this group, a high increase of the content of *trans*-piceatannol (633±64 mg/kg DW) and *trans*-ε-viniferin (2193±213 mg/kg DW) was detected even on the cultivar Bianchetta (Fig. 2). Compared to Pinot noir, which reached after 6 weeks the maximum content of piceatannol and resveratrol among all varieties, Bianchetta and IM 13.0.25 were able to reach the same or higher quantities for both compounds after a storage of 12 weeks.
In grape canes harvested in December Pinot noir showed a very small accumulation of resveratrol and piceatannol, while again Verdiso and IM 13.0.25 were able to reach a high content of both compounds (Fig. 3) (Supplementary tables 2-5).
Our findings confirm the results previously found by GUERRERO *et al.* (2016), which highlight the importance of the cultivar in the accumulation of stilbenes in grape canes after pruning. In addition, for the first time, we demonstrated how the pruning time affects both the quantity and the accumulation rate of stilbenes in pruned canes.
The information on the maximum stilbene content recoverable from canes of different grapevine cultivars could be interesting for grape producers in order to obtain cane extracts with high stilbenes concentration from their own grape canes waste. These extracts can be the base for the purification of stilbenes to be used in the food or cosmetic industries, with a big economic income considering the value of food-grade resveratrol is about 2000-3000 US$/kg (ZHANG *et al.*, 2011). However, the crude cane extract could be also reused by grape growers in the same vineyard in an idea of circular economy. In fact, stilbenes have shown antifungal activity against different fungi. Until now, the antifungal activity *in vitro* of the crude cane extracts from Pinot noir, Gamaret and Divico cultivars against *Plasmopara viticola, Erysiphe necator* and *Botrytis cinerea* was reported by SCHNEE *et al.* (2013). Recently, the direct antifungal activity of crude cane extract from Pinot noir against *B. cinerea* was studied by monitoring the mycelium growth on nutrient agar medium, and also in grapevine plants *in vitro* and *in vivo* by DE BONA *et al*. (2019).

4. CONCLUSION

The information on the maximum stilbene content recoverable from canes of different grapevine cultivars could be interesting for grape producers, but many factors have to be taken in to account to obtain the highest yield of these compounds. First, the storage time is the main factor driving the increase of stilbenoids in grape canes, confirming the literature data, but we demonstrated for the first time that the total amount of stilbenoids and their rate of accumulation depends significantly on the pruning time. In addition the data reported in the present study confirm the importance of the cultivar in the accumulation of stilbenes in grape canes after pruning. Finally, this study showed that the cultivars Verdiso and Incrocio Manzoni 13.0.25 possess a high potential of stilbene accumulation, mainly when the canes were harvested in October.

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