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Yield reduction through cluster or selective berry thinning similarly modulates anthocyanins and proanthocyanidins composition in Refosco dal peduncolo rosso (*Vitis vinifera* L.) grapes

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ABSTRACT

'Refosco dal peduncolo rosso' is a late-ripening and low-yielding red grape variety, mainly cultivated in North-eastern Italy (Veneto and Friuli Venezia Giulia), and characterized by relatively high anthocyanins and average-to-low tannins concentrations. Under Friuli's climatic conditions, it is often challenging to maintain the Refosco dal peduncolo rosso grapes hanging on the vines long enough to match complete berry maturation before the rain season starts. Therefore, winegrowers normally perform cluster thinning in order to enhance or accelerate ripening. This study compared the effects of selective berry thinning (cluster shoulders and tips removal) and classical cluster thinning on fruit technological maturity, anthocyanin profile, and skin and seed proanthocyanidins concentration. Our results revealed that both thinning treatments induced a significant increase in total soluble solids, as well as total anthocyanins through the specific enhancement of OH- and di-substituted monoglucosylated anthocyanins. Additionally, skin high molecular weight proanthocyanidins was reduced by selective berry thinning, while mean degree of polymerization and percentage of galloylation were significantly decreased by both thinning treatments. These results showed that the yield reduction obtained by both methods were profitable to improve the maturation of Refosco dal peduncolo rosso grapes. However, the application of selective berry thinning provided a significant reduction of both skin high molecular weight proanthocyanidins and percentage of prodelphinidins. Therefore, the first evidences on grapes composition favored the cluster thinning technique as less time consuming, but more research on wine and sensory effects is needed to confirm the potential of selective berry thinning.

1. Introduction

Within the autochthonous red varieties cultivated in North-Eastern Italy, 'Refosco dal peduncolo rosso' is the most important in the Friuli Venezia Giulia (FVG) region. This late-ripening and low-yielding genotype accumulates high anthocyanins concentration and a mean-to-low amount of tannins (Costacurta et al., 2005). However, under the FVG climatic conditions not all the seasons allow a good maturation of the grapes of Refosco dal peduncolo rosso, thus winegrowers normally perform cluster thinning with the aim to obtain a better or faster maturation.

Crop thinning is a common viticultural practice widely used to adjust vine yield and mainly performed to equilibrate vines with an excessive crop level in comparison to its vegetative capacity (such as in high-yielding cultivars; e.g. Sangiovese, Montepulciano, Trebbiano, etc.). It is assumed then that by improving the leaf area-to-fruit ratio, the fruit reaches a proper level of maturity (Frioni et al., 2017; Kliewer and Dokoozlian, 2005). This in turn, foregoes the negative consequences of excessive delays in ripening (e.g. start of the rainy season

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that increases the risk of bunch rots) in case of late-ripening grapevine varieties (Guidoni et al., 2008; Palliotti et al., 2014; Palliotti and Cartechini, 2000). Several studies have confirmed the positive effect of cluster thinning on grape and wine quality, with particular regard to soluble solids and anthocyanins content, for a wide range of cultivars and growing conditions (Dami et al., 2006; Guidoni et al., 2008; King et al., 2015; Xi et al., 2018). From a chemical and sensory perspective, the two most important phenolic classes contributing to the quality of red grapes and wines are anthocyanins and proanthocyanidins. Previous studies showed that cluster thinning can affect the substitution pattern of anthocyanins, and thinned vines of 'Cabernet Sauvignon' and 'Sangiovese' varieties showed a shift in flavonoid metabolism leading to significantly higher di-substituted anthocyanins (Pastore et al., 2011; Wang et al., 2018). However, very few experiments evaluated the impact of thinning on grape proanthocyanidins. Lower concentration of polymeric condensed tannins was found in seeds, skins and pulp from cluster-thinned vines, in Cabernet Sauvignon and Tempranillo (do Ó-Marques et al., 2005), while no effects on proanthocyanidins concentration in skin or seeds were found in Malbec (Fanzone et al., 2011).

Selective berry thinning (removal of the cluster parts such as the bottom tips), has been proposed as alternative to traditional cluster thinning to improve grape maturity (Molitor et al., 2012; Roberto et al., 2015) despite being more labor intensive and less economically sustainable (Gatti et al., 2012; Palliotti and Cartechini, 2000). This technique is based on the observation that for some cultivars (such as Cabernet Sauvignon) the berries located in the tips of clusters ripen later than in the body or shoulders (Tarter and Keuter, 2005; Winkler et al., 1974). A study in 'Brancellao' showed that berries in the shoulder reached maximum anthocyanin levels 30 days earlier than those from the tip, even if no differences were observed either in anthocyanins nor in flavonols at harvest (Figueiredo-González et al., 2012). Gil et al. (2013) indicated that, in Syrah, wines produced from cluster thinned vines had significantly higher anthocyanins than wines from the control, whereas berry thinned (tip removal) vines made wines containing more flavonols and proanthocyanidins when compared to cluster thinning and the control vines. In the same work, selective tips removal led to higher proanthocyanidin mean degree of polymerization (mDP) and concentration in wines when compared to cluster thinning.

While the beneficial effects of the adoption of crop thinning techniques in high-yielding grape varieties appears clear, little detailed reports are available for its application on low-yielding varieties such as the case of Refosco dal peduncolo rosso. Nevertheless, local grape growers' empirical knowledge advocates for positive impacts. This study was designed to compare the effects of selective berry thinning (shoulders and tips removal) and classical cluster thinning on fruit technological maturity, anthocyanin profile, and skin and seed proanthocyanidins concentration of Refosco dal peduncolo rosso grapes. Additionally, we studied the variability of ripening initiation (veraison) by observing the progression of color change on berries from the shoulders, tips and main body of non-treated vines.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, acetone, absolute ethanol of HPLC grade, glacial and hydrochloric acid of ACS purity, phloroglucinol, L-ascorbic acid and sodium acetate were from Sigma-Aldrich (Germany). Folin-Ciocalteu, iron (II) sulfate heptahydrate and vanillin were from Merck (Darmstadt, Germany). LC–MS methanol (Chromasolv, Honeywell Riedel-de Haën™, Germany) and ultra-pure water of Milli Q gradient (Millipore Corporation, Billerica, MA, USA) were used for UHPLC-DAD-MS/MS.

2.2. Vineyard site, vine material and experimental design

The experiment was carried out during 2016 and 2017, in a commercial vineyard located in Pavia di Udine (North Eastern Italy, Lat. 45° 57′ 52″ N, Long. 13° 17′ 55.18″ E, Altitude: 52 m asl), within the D.O.C. area Friuli Grave. The vineyard soil is a cutani-profondic-luvisol, really deep (0.12-0.15 m), and characterized by a negligible coarse and a fine texture (25 % sand, 50 % silt and 25 % clay). Going from the superficial to the deeper horizons, the soil analysis provided by the owner showed no total and active limestone, a pH = 6.1, a cation exchange capacity and a percentage of organic matter ranging from 11.5 and 13.6 meq/100 g and from 1.1 and 0.5 %, respectively. Vines were planted in 2006 with 'Refosco dal peduncolo rosso' (clone ERSA FVG 401) grafted on SO4 rootstock, with an East-West row orientation, and spacing of 2.3 m between rows and 0.85 m within the row (5.600 vines/ha). Vines were trained as single bended Guyot with a fruiting cane pruned to 7–8 buds/vine, vertical shoot-positioned with a set of two double catching-wires, allowing a vertical canopy development of 1.2 m. During the growing seasons, shoot trimming was performed two times (post-flowering and pre-veraison), while leaf removal was not performed, since Refosco dal peduncolo rosso grapes are particularly sensitive to sunburns.

A completely randomized design with three treatments and three replication per treatment was established within three adjacent rows. In each row, three plots of 12 homogeneous vines were selected and tagged. One of three treatments were randomly assigned and imposed within each plot: C, untreated control; CT, Cluster Thinning (removal of entire clusters); ST, Selected cluster thinning (removal of the shoulders and tips in each cluster). Both CT and ST were applied at EL 34 pre-veraison stage (Lorenz et al., 1995) on 26 Jul 2016 and 24 Jul 2017, respectively, and produced a similar yield reduction at harvest (ca 47–50 %). In 2016, a preliminary test was carried out on 20 guard vines in order to calibrate the ST and CT treatments and to obtain a similar yield reduction between vines. ST was performed removing tips and shoulders of all clusters within the experimental plots. As regard CT, the second cluster of the shoot was removed when present, and thereafter one cluster every 2–3 shoots preferring to eliminate some of the big clusters that grouped together at the top of the bent cane, to improve also microclimate and avoid bunch rots.

On July 20 of both seasons, 15 clusters were selected on untreated vines, and 6 berries were tagged on the body, the shoulders, and the tips, to monitor veraison color-change dynamics in the different parts of the clusters every 1–2 days as described in Herrera and Castellarin (2016).

Meteorological data were recorded by the ARPA-OSMER weather station of Lauzacco (ARPA FVG–OSMER, http://www.meteo.fvg.it/), located 3 km from the vineyard.

In general weather conditions were similar during the two experimental years. From April 1 till harvest, the mean temperature was comparable between the seasons, while the rain was lower in the 2016 (607 mm) as compared to 2017 (746 mm) showing also different distribution patterns along the growing season (Fig. 1).

In the first part of the season, temperatures were relatively similar between years, while from May 11 until the end of June, temperatures were higher in 2017 (meanly +2.2 °C) and the rain much lower. Additionally, from August 20 to the beginning of October, temperatures were lower in the season 2016 (meanly -2.1 °C), and between September 1 and harvest time rain was nearly double in 2017 (214 mm) than in 2016 (102 mm). From April 1 until harvest, cumulated growing day degrees (GDD) were 1742 and 1740 in 2016 (September 19) and 2017 (September 14), respectively.

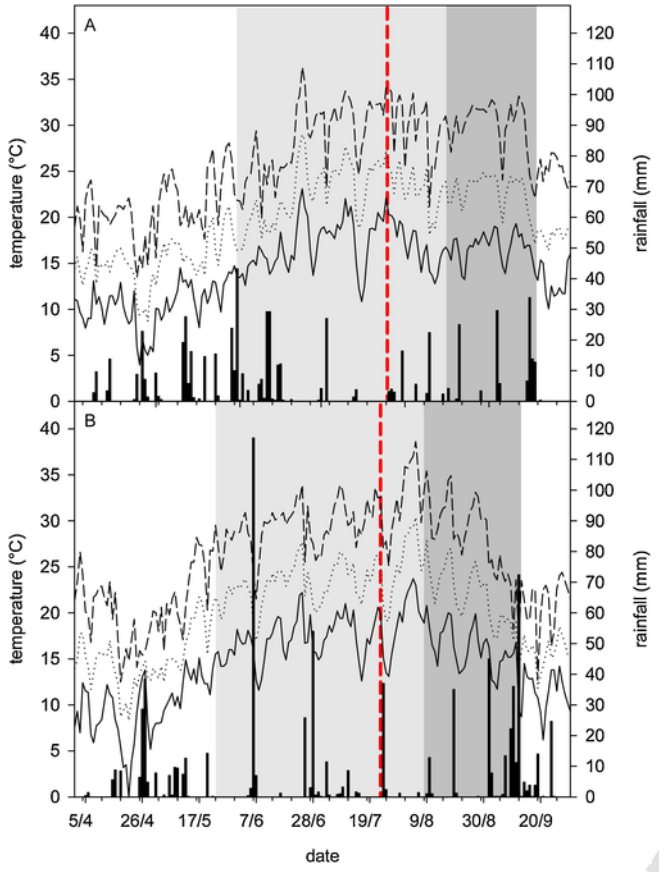

Fig. 1. Trends of mean, min and max temperatures and rain during the seasons 2016 (A) and 2017 (B). Data recorded at the weather station of Lauzacco (ARPA FVG–OSMER, http://www.meteo.fvg.it/). Pale grey indicates the period between flowering (EL 23) and veraison (EL 35), while dark grey between veraison and harvest (EL 38). The dotted red line highlights the time of thinning applications. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Leaf area, yield components, basic analysis of grape juice

At harvest, three representative shoots per replicate were collected and leaf area was measured in the laboratory using a leaf-area meter (LI-3100, LI-COR, Lincoln, NE). Primary and lateral leaf area was kept separated. The number of shoots was determined on all the experimental vines to calculate vine leaf area by multiplying the average shoot leaf area per the total number of shoots per vine. All vines were hand harvested on 19 September 2016 and 14 September 2017, respectively. Yield and cluster number per vine were recorded and the average cluster weight was calculated. At harvest, two sets of 40 berries were sampled. The first set of samples was hand squeezed and the grape juice was used to determine basic technological parameters. Total soluble solids (°Brix) and pH were determined using a manual refractometer (ATC-1, Atago, Tokyo, Japan), and a pH meter (HI2211, Hanna Instruments, Woonsocket, RI). Titratable acidity (expressed as g/L tartaric acid equivalents) was determined by titration of the juice with NaOH 0.1 N until pH 8.2. The second set of 40 berries was immediately stored at −80 °C for further analysis.

2.4. Processing of seed and skin samples and preparation of extracts

The 40 berries stored at −80 °C were counted and weighed, and the skins and seeds were separated from the flesh while kept on ice. Recovered skin and seed material was weighed to determine the proportion of fresh skin and seed material to total berry mass. Skin and seed tissues were ground into a fine powder in presence of liquid nitrogen, with an A11B IKA analytic mill (Königswinter, Germany) and stored in 15 mL tubes.

Anthocyanins were extracted by adding 0.18 g of fresh skin powder and an aliquot of 1.8 mL of methanol in water in a 1:1 ratio (v/v) in a 2 mL microtube. The extraction was performed at room temperature in an ultrasonic bath for 1 h. Samples were then centrifuged at 15,000 rpm for 15 min, diluted, and filtered using 0.2 μm regenerated cellulose membranes (Phenomenex, Castelmaggiore, Italy). Samples were stored in HPLC vials at −20 °C before analysis.

The extraction of proanthocyanidins from skins and seeds were carried out according to Chira et al. (2009) with slight modifications. Seed and skin powder was lyophilized for two days and stored at −80 °C. The frozen seed and skin powder was extracted in 1:9 (w/v) of acetone:water (80:20, v:v) for 4 h, centrifuged at 5,000 rpm for 15 min at 4 °C. Sediment was then extracted in the same volume of methanol:water (60:40,v:v) for 3 h and centrifuged again. Acetone and methanol extracts were pooled and evaporated under reduced pressure at 38 °C to remove organic solvents. Residue was re-dissolved in water and freeze-dried to obtain a crude tannin extract.

2.5. HPLC-DAD determination of anthocyanins in grapes

Anthocyanin concentration and profile were determined by HPLC (LC-20AT, Shimadzu, Kyoto, Japan) equipped with a diode array detector (SPD-M 20 A, Shimadzu). Separation was performed using a C-18 column (LiChroCART 250–4, Merck, Darmstadt, Germany) maintained at 25 °C. The solvents used, the gradient and all the other chromatographic conditions are reported in Sivilotti et al. (2016) The concentration of individual anthocyanins was expressed in oenin chloride equivalents as mg/g of fresh berries or as mg/g of fresh skins.

2.6. Spectrophotometric determination of low and high molecular weight tannins

Low and high molecular weight proanthocyanidins in seed and skin crude tannin extract were analyzed as described by Rigo et al.(Rigo et al., 2000) Analysis was performed with an Agilent 8453 spectrophotometer (Agilent technologies, Palo Alto, USA). 10 mg of freeze-dried skin or seed tannin powder was dissolved in 1 mL of methanol. Samples were than further diluted in methanol approximately 10-to-25 times. The dilution factor was adjusted to obtain a final reading between 0.3 and 0.6 AU.

2.7. Low molecular weight proanthocyanidins (LMWPs)-index of vanillin

LMWPs were determined through catechins and proanthocyanidins reactive to vanillin. 1 mL of skin or seed extract in MeOH was placed into a 50 mL flask (shielded from light) containing 6 mL of 4 % vanillin (in MeOH). Exactly 15 min after 3 mL of concentrated HCl was added, the absorbance was measured at 500 nm in a 10 mm cell against a blank prepared in the same conditions, containing MeOH instead of vanillin. The method provides estimation of free C6 and C8 of both catechins and proanthocyanidins. This index decreases with an increase in polymerization, because C6 and C8 are mainly involved in polymerization bonds. The method provides a good estimation of free flavanols and a low degree of polymerized flavanols. LMWPs were evaluated as (+)-catechin in mg/g of berry FW.

2.8. High molecular weight proanthocyanidins (HMWPs)

3 mL of skin or seed extract in MeOH were placed in a 50 mL flask shielded from light (aluminum foil) and containing 9.5 mL absolute ethanol. 12.5 mL of $FeSO_4 \times 7H_2O$ (300 mg/L) in concentrated HCl was

added, and the flask was placed in a boiling water bath and refluxed for 50 min, after which time it was rapidly cooled by immersion in cold water (20 °C). HMWPs were evaluated by transformation into cyanidin and expressed in mg/g of cyanidin chloride berry FW. The method provides a good estimation for evaluation of high polymerized tannins (Vrhovsek et al., 2001).

2.9. UHPLC-DAD-MS/MS analysis of proanthocyanidin structural characteristics

The structural characteristics of proanthocyanidins, comprising mean degree of polymerization (mDP), percent of galloylation (%G) and percent of prodelphinidins (%P) were determined after acid-catalyzed degradation with phloroglucinol as nucleophilic reagent (Drinkine et al., 2007; Kennedy and Jones, 2001). Briefly, crude freeze-dried seed or skin extract was diluted in methanol (5 mg/mL) and mixed 1:1 (v/v) with a solution of 0.05 M HCl in methanol containing 50 g/L phloroglucinol and 10 g/L ascorbic acid. The mixture was held at 50 °C for 20 min to allow fractionation. After 20 min, 5 volumes of 40 mM aqueous sodium acetate were added to stop the reaction. Samples were filtered through a 0.22 μm PVDF filter from Millipore (Billerica, MA, USA) and analyzed using a 1290 infinity UHPLC system coupled to an ultrasensitive DAD detector (G4212A) and a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, USA). Samples were kept at 4 °C during analysis and the injection volume was 10 μL. Separation was performed on a 100 mm x 4.6 mm, 3.5 μm column (XTerra RP18, Waters). Flow was set to 0.7 mL/min, mobile phase A was 1 % (v/v) aqueous acetic acid and B methanol.

Separation was carried out at 40 °C using a linear gradient starting at 5 % B for 25 min, from 5 % to 20 % B in 20 min, from 20 % to 32 % B in 15 min and from 32 % to 100 % B in 2 min; 100 % B for 5 min, to 5 % B in 1 min and 5 % B for 4 min. The UV–vis spectra were recorded from 210 to 400 nm with detection at 280 nm. Total ion chromatograms (TICs) were monitored in negative ion mode using electrospray ionization mass spectrometry (ESI, Jet-Stream). Nitrogen was used as the carrier gas and the source parameters were: gas temperature 250 °C, gas flow 6 L min^{-1}, nebulizer 241 kPa, sheath gas heater 375 °C, sheath gas flow 10 L min^{-1}, capillary voltage 3500 V. Identification of flavan-3-ols and their phloroglucinol adducts was performed using the molecular ion $((M-H)^{-}$, which was *m/z* 289 for catechin and epicatechin; *m/z* 305 for gallocatechin and epigallocatechin; *m/z* 441 for epicatechin gallate; *m/z* 413 for catechin- and epicatechin-phloroglucinol, *m/z* 429 for epigallocatechin-phloroglucinol and *m/z* 565 for epicatechin gallate-phloroglucinol. mDP, %P and %G were estimated using the response factors of PA cleavage products at 280 nm and calculated as described by Kennedy and Jones (2001) The mDP value represented the molar ratio between the sum of all flavan-3-ol units produced by phloroglucinolysis and the sum of terminal units.

2.10. Statistical analysis

Data were analyzed using two-way ANOVA with treatments and year as fixed factors; when the differences were significant, means were separated with Student Newman Keuls's test ($p < 0.05$).

The effect of selected berry thinning on the speed of the pigmentation was assessed using a survival analysis technique (Herrera and Castellarin, 2016). Survival analysis is commonly used to study follow-up times from a defined starting point to the occurrence of a given event. We used this method to calculate the survival function for the whole cluster and for the cluster without tips and shoulders testing the significance of differences using the χ^2 test ($p < 0.05$).

Both ANOVA and survival analysis were performed using 106 JMP® software (JMP 7.0, SAS Institute Inc., NC, USA). Figures were created using SigmaPlot 13 (Systat Software GmbH, Erkrath, Germany).

3. Results

3.1. Veraison asynchrony tips-body-shoulders

Veraison time (50 % color change) was advanced by one week in the second year of the trial, occurring on August 14 in 2016, 78 Days After Anthesis (DAA) – and August 7 in 2017 (74 DAA). The average temperature of the last two weeks before veraison was 22.2 °C and 25.5 °C in the seasons 2016 and 2017, respectively, and this difference could help explaining why veraison was reached earlier in 2017. As a result, the progression of berry coloring and the asynchrony between the central part of the cluster, the shoulders, and the tips was different. During 2016, the first colored berries appeared on August 9 in the cluster body, followed by the shoulders one day later and tips on August 12. As already reported above, mid-veraison in the cluster body and shoulders was detected on August 14 and five days later on cluster tips. Finally, cluster body and shoulders reached the end of veraison (100 % coloration) on August 24 and 25, respectively, while the tips 3 days later. A similar trend was observed in 2017, with the first colored berries appearing on July 29 in the cluster body, on July 31 in the shoulders, and on August 2 in the tips. Interestingly, in 2017 the berries on cluster shoulders reached mid-veraison 4 days earlier than cluster body and tips. The cluster body ended veraison on Aug 17, whereas the shoulders and tips were delayed by 2 and 7 days, respectively. The survival curves (Fig. 2) describe the disappearance of green berries (or the progression of veraison) in berries of the cluster bodies and tips during veraison, showing how the latter curve was significantly delayed in both seasons. In contrast, when cluster body and shoulders are compared, the survival curves were nearly overlapped in both seasons, and no significant differences between the models were revealed.

3.2. Yield components and basic fruit chemistry parameters

The effects of the thinning treatments on yield parameters, berry parameters and technological maturation are shown in Table 1.

As expected, the number of clusters was significantly reduced only by CT, whereas both CT and ST significantly decreased the average cluster weight. The lower cluster weight of CT treatment can be ascribed to the targeted removal of big clusters during thinning (see Materials and Methods). The yield per vine was significantly lower in CT (-46 %) and ST (-48 %) compared to control vines and can be explained by the two parameters above described. Yield parameters were also affected by the season; the higher rainfall in 2017 likely influenced the average cluster weight and yield.

The total leaf area and individual components (main leaf area, lateral leaf area), were not affected by the treatments. This result was expected because treatments were performed at veraison, which occurs after canopy growth ceases. The leaf area-to-fruit ratio (LA/F) in C, CT and ST accounted for 1.90, 3.34 and 3.15 m^2/kg, respectively, far above the optimal range of 0.7-1.4 m^2/kg proposed by Kliewer and Dokoozlian (2005).

Berry components analysis (Table 1) reveals that CT significantly increased the skin mass per berry (mg/berry, +13.3 %) and the relative skin mass per berry (mg/g berry, +21.1 %) as compared to C, while no differences were shown for ST.

Sugar accumulation was evaluated as total soluble solids (°Brix), and was significantly increased in both thinning treatments compared against the control (19.4, 21.0 and 21.6°Brix in C, CT, and ST, respectively), but in contrast, no significant differences were detected between ST and CT (Table 2). Both titratable acidity and pH were not in-

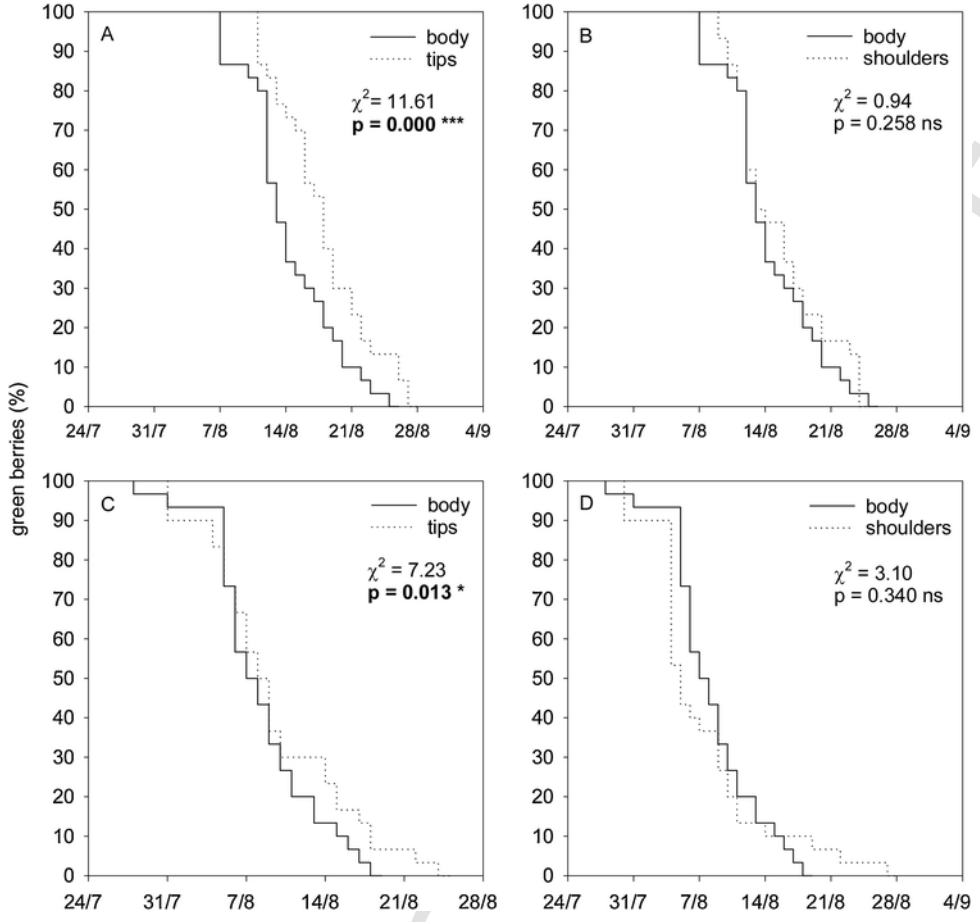

Fig. 2. Kaplan-Meier survival curves comparing the disappearance of green berries in body vs tip (A, C) and body vs shoulders (B, D) in 2016 (A, B) and 2017 (C, D). Significant differences between comparisons are shown (ns, not significant; *, $p < 0.05$; ***, $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Yield and berry components of Refosco dal peduncolo rosso subjected to cluster thinning treatments in the seasons 2016 and 2017.

	Treatment (T)				Year (Y)			T x Y interaction
	C	CT	ST	sign. F[a]	2016	2017	sign. F	sign. F
YIELD PARAMETERS								
Yield (kg/vine)	2.31 a[b]	1.25 b	1.20 b	***	1.21 b	1.98 a	***	*
N° clusters	11.63 a	7.79 b	10.67 a	***	10.15	9.97	ns	ns
Average cluster weight (g)	196.35 a	162.22 b	113.57 c	***	119.42 b	196.11 a	***	*
Leaf area main shoots (m^2/vine)	1.79	1.92	1.82	ns	1.73 b	1.98 a	**	ns
Leaf area laterals (m^2/vine)	1.98	2.09	1.92	ns	1.54 b	2.54 a	***	ns
Total leaf area (m^2/vine)	3.77	4.01	3.74	ns	3.27 b	4.52 a	***	ns
Leaf area-to-fruit (m^2/kg)	1.90 b	3.34 a	3.15 a	***	2.85	2.62	ns	ns
Average berry weight (g)	1.73	1.63	1.67	ns	1.48	1.87	***	ns
Average skin weight (mg/berry)	116.54 b	132.10 a	120.44 a	*	110.66 b	135.39 a	***	ns
Average seeds weight (mg/berry)	93.86	90.76	92.14	ns	82.40 b	102.10 a	***	ns
Skin-to-berry ratio (mg/g berry)	67.34 b	81.54 a	72.23 b	***	74.89	72.52	ns	ns
Seed-to-berry ratio (mg/g berry)	54.44	55.85	55.24	ns	55.70	54.65	ns	ns

[a] Data were analyzed through two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).

[b] Different letters (a, b, c) identify significantly different means. C, untreated control; CT, cluster thinning; ST, selective cluster thinning. (n = 3; yield, cluster number and weight and leaf area was evaluated on 10 vines/replicate; berry parameters were evaluated on the 40 berries/replicate used for polyphenolic analysis).

Table 2
Basic and polyphenolic grape composition of Refosco dal peduncolo rosso subjected to cluster thinning treatments in the seasons 2016 and 2017.

	Treatment (T)				Year (Y)			T x Y interaction
	C	CT	ST	sign. F[a]	2016	2017	sign. F	sign. F
BASIC GRAPE COMPOSITION								
Total soluble solids (°Brix)	19.4 b[b]	21.0 a	21.6 a	*	21.7 a	19.6 b	***	ns
Titratable acidity (g/L)	6.39	6.02	6.24	ns	7.28 a	5.16 b	***	ns
pH	3.31	3.36	3.35	ns	3.34	3.34	ns	ns
ANTHOCYANINS								
Total anthocyanins (mg/g berry FW)	3.03 b	3.99 a	3.78 a	*	3.83	3.37	ns	ns
Total anthocyanins (mg/g skin DW)	84.33	86.82	98.88	ns	93.60	85.09	ns	ns
OH-substituted forms (%)	20.14 b	22.87 a	22.42 a	**	21.07 b	22.93 a	**	ns
OCH_3-substituted forms (%)	79.86 a	77.13 b	77.58 b	**	78.93 a	77.07 b	**	ns
Di-substituted forms (%)	18.36 b	20.32 a	19.87 a	**	18.09 b	21.41 a	***	ns
Tri-substituted forms (%)	81.64 a	79.68 b	78.93 b	**	81.91 a	78.59 b	***	ns
Mono-glucosylated forms (%)	55.85 b	58.94 a	58.86 a	**	58.00	58.10	ns	ns
Acetyl-glucosylated forms (%)	25.03	24.34	24.33	ns	24.58	24.47	ns	ns
p-coumaryl & caffeoyl-glucosylated forms (%)	19.13 a	16.81 b	16.81 b	**	17.42	17.43	ns	ns
SKIN PROANTHOCYANIDINS								
LMWP (mg/g berry FW)	1.96	1.63	1.41	ns	1.72	1.39	ns	ns
HMWP (mg/g berry FW)	5.11 a	5.35 a	4.13 b	*	3.95 b	5.77 a	***	**
mDP	18.8 a	16.2 b	15.9 b	*	20.46 a	13.49 b	***	ns
% prodelphinidins	34.5 a	35.0 a	32.3 b	*	38.35 a	29.47 b	***	ns
% galloylation	13.3 a	10.9 b	11.7 b	*	9.33 b	14.6 a	***	ns
SEED PROANTHOCYANIDINS								
LMWP (mg/g berry FW)	1.06	0.97	1.06	ns	0.97	1.10	ns	ns
HMWP (mg/g berry FW)	1.25	1.19	1.19	ns	1.16	1.27	ns	ns
mDP	10.20	9.89	9.49	ns	9.81	9.88	ns	ns
% galloylation	49.91	50.95	51.98	ns	48.62 b	53.69 a	***	ns

[a] Data were analyzed through two-way ANOVA (ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), and when differences were significant, the means were separated using Student Newman Keuls test ($p < 0.05$).

[b] Different letters (a, b) identify significantly different means. C, untreated control; CT, cluster thinning; ST, selective cluster thinning; LMWP and HMWP, low and high molecular weight proanthocyanidins; mDP, mean degree of polymerization; DW, dry weight; FW, fresh weight. (n = 3; for each replicate 80 berries were sampled on 10 vines, 40 berries used for basic analysis and 40 for polyphenolic analysis).

fluenced by CT or ST and values were standard for the area at harvest (ca. 6.0 g/L acidity and pH of 3.3).

3.3. Anthocyanins content and substitution patterns

Skin anthocyanins were analyzed and identified in harvest samples from both experimental years. Anthocyanin profiles of 'Refosco dal peduncolo rosso' grapes were characterized by sixteen forms, including the 3-O-monoglucosides of cyanidin, peonidin, delphinidin, petunidin, and malvidin, their relative acetyl and coumaroyl esters, and the peonidin caffeoyl-glucoside (Table 2, Fig. 3).

Total anthocyanins concentration (mg/g berry) was significantly higher in ST (3.78 mg/g) and CT (3.99 mg/g) vines than in C (3.03 mg/g), and a similar trend was also observed for anthocyanin content (expressed in relation to skin weight) but without significant differences between treatments.

For the anthocyanins data, no significant interaction was found analyzing year and treatments together. For this reason, data from 2016 and 2017 was pooled together for evaluation of anthocyanins substitution pattern (Table 2). When single anthocyanins concentrations were analyzed as fold changes from untreated control, the thinning treatments exhibited a clear shift in anthocyanins profile (an increase in almost all anthocyanins), more evident when the data related to berry fresh weight were considered (Fig. 3). All monoglucoside-anthocyanins showed an increase due to thinning treatments, with the di-substituted and hydroxylated forms and their derivatives reporting a significant increase (Table 2, Tab. S1). When anthocyanins were reported in relation to berry weight, they were all increased by both the thinning treatments with the exception of peonidin-coumaryl-3-glucoside and malvidin-3-monoglucoside and derived esters (Tab S1).

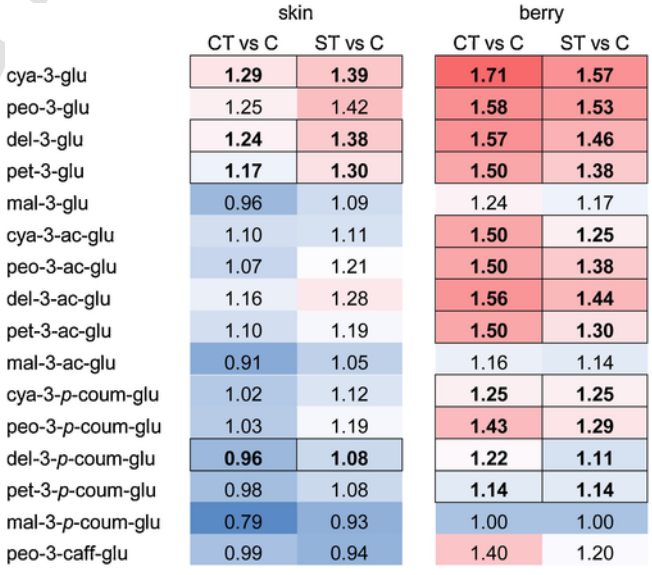

Fig. 3. Heat map representation of anthocyanins identified in Refosco dal peduncolo rosso grapes (mean 2016 and 2017) as fold change from the control treatment. Rates of skin concentrations (DW) and berry concentrations (FW) are reported in the left and right part of the graph, respectively. Red and blue colors indicate high and low percentage change of metabolite, respectively. Bolded, outlined values represent significant changes from the control, $p < 0.05$. C, control; CT, cluster thinning; ST, selective cluster thinning. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Total skins and seeds tannins and their composition

The concentration of both HMWP and LMWP (mg/berry FW) proanthocyanidins was higher in skins than in seeds in both investigated vintages, regardless of treatment (Table 2). When comparing the treatments, the skin concentration of HMWP was significantly reduced in case of ST, while no differences were observed for LMWP. However, a significant interaction between year and treatment was observed for this parameter, revealing that in the second season, negligible effects of treatments were found as compared to C. The mDP of skin proanthocyanidins was 18.8 in case of C and was significantly lower for both CT (16.2) and ST (15.9) thinning treatments, with no differences between them. The same differences were found also regarding the % of galloylation, while only ST showed significantly lower values of % of prodelphinidines as compared to C and CT treatments.

The proanthocyanidin (LMWP and HMWP) concentration in the seeds was not significantly modified in 'Refosco dal peduncolo rosso' by either thinning approach. In addition, also seed structural characteristics of tannins (mDP and % galloylation) did no display remarkable difference in our experiment. ST and CT showed slightly lower mDP values than C, but the variation was not significant.

4. Discussion

Our experiment results showed that both thinning techniques positively influenced the berry composition at harvest by enhancing the sugar accumulation and anthocyanins concentration. Previous research showed similar results (Guidoni et al., 2008; Karoglan et al., 2014; King et al., 2015). Our observations on the progression of veraison (color change) in Refosco dal Peduncolo Rosso highlighted that cluster tips in both years were significantly in delay as compared with the other portions of the clusters, while the coloring of shoulders and cluster body was nearly similar and no significant difference was found (Fig. 2). Some authors showed that berries from the tip of the cluster are generally lower in soluble solids than those from the shoulder or mid-cluster regions (Kasimatis et al., 1975), because they are a subordinate priority for photo assimilate supply (Kasimatis et al., 1975; Pagay and Cheng, 2010). On the other hand, sugars are known as signaling molecules for anthocyanin biosynthesis and accumulation (Gouthu and Deluc, 2015). Although we did not measure sugar accumulation in the different cluster parts, it is likely that berries from tips were less competitive for sugars, and this caused the delay in anthocyanins accumulation and veraison progression time. Such result would advocate for the effectiveness of selective berry thinning on the ripening enhancement found here, but would also implicate that classical cluster thinning has an effect on the ripening differences along the cluster. Because there is evidence of metabolic synchronization between the different parts of the cluster towards maturity and harvest (Dal Santo et al., 2013; Gouthu and Deluc, 2015), it is more plausible that source-sink balance mechanisms were more important players than the heterogeneity of berry ripening within single clusters. Indeed, increased sugar concentrations in grape juice can typically be attributed to a reduction in yield (Intrieri et al., 2004) or to an increase in the LA/F (Kliewer and Dokoozlian, 2005; Pastore et al., 2011), parameters which were both altered in this experiment. Our results showed that both CT and ST resulted in a significant increase of soluble solids as compare to C although all the treatments could be considered "under-cropped" (LA/F > 1.9 m^2/kg). The LA/F reference values of 0.7-1.4 m^2/kg (Kliewer and Dokoozlian, 2005) were identified in a climate completely different from Northern Italy and previous trials carried out on Merlot (Sivilotti et al., 2016), Sauvignon blanc (Sivilotti et al., 2017), and Nebbiolo (Guidoni et al., 2008), pointed out that maybe a different range of crop load should be adopted. In our experimental conditions, it should be also taken into account that the vineyard planting was rather dense (0.85 m space between vines) resulting in middle shading conditions for most of the leaves, with obvious consequences on their photosynthetic efficiency and net assimilation balance. Under such conditions, accounting for all the leaves of the canopy (as we did here), might have biased the LA/F ratio significance in terms of source-sink balance. Other studies dealing with cluster thinning showed that the increase of the leaf area-to-yield ratio was beneficial to improve maturation (Bubola et al., 2017; Palliotti and Cartechini, 2000), and that an increase of the ratio above the optimal range increased anthocyanins concentration, while not affecting soluble solids.

Anthocyanins concentration was significantly higher in ST and CT vines in comparison to C and previous studies have reported similar positive effect of thinning on the berry anthocyanins and phenolics (Gatti et al., 2012; Guidoni et al., 2008; Pastore et al., 2011; Petrie and Clingeleffer, 2004). The enhancement of anthocyanins concentration is often related to higher soluble solids or hexoses concentrations in berries from thinned vines (Bubola et al., 2011; Pastore et al., 2011; Xi et al., 2018, 2016); however, in our experiment, it is likely that the increased proportion of skin per berry in both CT and ST accounted for the enhanced concentrations (mg/g berry), as no effect was observed in the concentration of anthocyanins per gram of skin (mg/g skin). On the other hand, Poni et al.(2018) and Suklje et al. (2013) reported that the accumulation of secondary metabolites could be decoupled form sugar accumulation leading to higher concentration in a shorter time, or better grape quality at harvest.

When considering the anthocyanin profile, the percentage of hydroxylated, disubstituted and monoglucosilated anthocyanins was improved by both CT and ST thinning treatments. Malvidin has been recognized as the most stable of the anthocyanin compounds found in grape, and is the least sensitive to changes in environmental conditions (Vanderweide et al., 2018). Our results are in agreement with previous studies showing that cluster thinning could stimulate the biosynthesis of non-methoxylated rather than methoxylated anthocyanins (Pastore et al., 2011; Wang et al., 2018). Similarly to what has been reported by other authors (Bubola et al., 2011; Guidoni et al., 2008), a significant increase in monoglucoside anthocyanins was found in the berries from thinned vines, even if the concentration of the malvidin-3-monoglucoside did not differ significantly among treatments. Related acylated forms reported the same increase, while a reduced impact on coumarylated and caffeoylated anthocyanins was detected. Similar to malvidin, acylated compounds are very stable, and unreactive to environmental conditions, while coumarylated anthocyanins have been shown to be reactive to light in other species (Tattini et al., 2014).

The significant interaction between year and treatment, observed in case of skin HMWP, revealed that significantly lower values of this parameter were found in case of ST only in the first season 2016, while higher and similar values were ascertained in C and CT. In the second season negligible effects of treatments were found. Cluster thinning experiments carried out on Cabernet Sauvignon and Tempranillo showed a reduction in the concentration of seed and skin polymeric tannins (do Ó-Marques et al., 2005), while opposite results were reported for Cabernet Sauvignon (Song et al., 2018). In our experiment, a combination of effects could explain the results obtained. The skin concentration of HMWP (in mg/g skin; data not reported) was significantly lower for both CT and ST treatments, suggesting a faster ripening, since proanthocyanidins can react with other cellular structures such as cell wall, polysaccharides, lignin and proteins (Downey et al., 2003; Hanlin et al., 2010). The higher skin-to-berry ratio observed in CT could partly explain the lack of significance with C; moreover, the elimination of tips in ST treatment probably contributed to reduce the concentration of HMWP, since tips are delayed in maturation and so could be responsible for higher amounts of HMWP.

Both CT and ST treatments promoted a significant reduction of mDP and of the percentage of galloylation in skins, while a decrease in the percentage of prodelphinidins was detected only for ST treatment. We already pointed out that more polymerized proanthocyanidins could interact with cellular structures, and probably these interactions increase when the concentration of proanthocyanidins is higher. In case of thinning we could speculate an increase of proanthocyanidins concentration and also their degree of polymerization, thus expecting a higher interaction with cellular structures and so a lower extractability. Therefore, the extractable fraction of proanthocyanidins could be characterised by a lower degree of polymerization. The differences between treatments in the percentage of galloylation and prodelphinidines are not easy to be explained and there is no information in literature helping to understand such behavior. Bindon et al. (2010) demonstrated that a fraction of high molecular mass tannins is non-extractable in acetone, since it may possibly be covalently bound to the cell walls. Moreover, the same authors pointed out that during ripening cell walls become more porous and this might facilitate the adsorption of tannins (Bindon et al., 2014). In our experiment, CT and ST grapes were advanced in maturation (higher TSS) and so a part of highly polymerized skin proanthocyanidins might have not been extracted by means of organic solvents. Moreover, most of the both galloylated subunits and prodelphinidins were found predominantly as extension units of the proanthocyanidin chain (data not shown). Since longer tannins might bind with cell wall material, the percentage of galloylation was lower in CT and ST probably due to their position in extension units. Similarly, the lowest percentage of prodelphinidins in ST could be due to the interaction between cell wall material and HMWP occurring in the cluster body (without tips) more advanced in ripening.

The seed proanthocyanidin concentration, the percentage of galloylation and the mDP were not significantly modified in Refosco dal peduncolo rosso by either thinning approaches. Accordingly, most of the studies dealing with seed proanthocyanidins analysis showed no effects of whatever treatment on such component (Bordiga et al., 2011; Chira et al., 2009; do Ó-Marques et al., 2005).

Undoubtedly, the proanthocyanidin composition in seeds and skins is an important issue that deserves substantial further research, since there is a lack of knowledge on the effects of agronomical techniques on these compounds that considerably affect the organoleptic properties of red wines.

5. Conclusions

Taken together these outcomes indicated that both thinning treatments (ST, CT) positively impacted the concentration and the composition of skin tannins. Consistently with the more advanced ripening stage, grapes from thinned vines displayed lower mDP and reduced percentage of galloylation, features that could have beneficial effects on the perceived astringency of wines. Out of the present results, there is no justification for selective berry thinning as compared to traditional cluster thinning, even if the differences in HMWP concentration and in the percentage of prodelphinidines suggest that the modification in the composition of proanthocyanidins could account for a change in the extractability and possibly in the sensorial properties of the wines. Further experiments should be performed to demonstrate the effectiveness of this techniques, considering also microvinifications and the analysis of the aroma compounds.

CRediT authorship contribution statement

Paolo Sivilotti: Conceptualization, Investigation, Data curation, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Rachele Falchi:** Investigation, Writing - original draft. **Joshua Vanderweide:** Supervision, Data curation, Writing - review & editing. **Paolo Sabbatini:** Supervision, Data curation, Writing - review & editing. **Marijan Bubola:** Writing - review & editing. **Andreja Vanzo:** Investigation, Resources, Writing - review & editing. **Klemen Lisjak:** Investigation, Resources, Writing - review & editing. **Enrico Peterlunger:** Investigation, Writing - review & editing. **Jose Carlos Herrera:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2019.109166.

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