UNIVERSITY OF NOVA GORICA
SCHOOL OF VITICULTURE AND ENOLOGY

CHANGE IN YIELD PARAMETERS AND GRAPE ANTHOCYANIN PROFILE OF *VITIS VINIFERA* L. 'REFOŠK' AS AFFECTED BY CLUSTER THINNING AND PRE-FLOWERING LEAF REMOVAL

DIPLOMA THESIS

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POVZETEK

Redčenje grozdja in odstranjevanje listov v območju grozdja pred cvetenjem sta zelo razširjeni ampelotehniki za nadzor kakovosti grozdja s pomočjo zagotavljanja ravnovesja med količino pridelka in listno površino. V tej diplomski nalogi smo prikazali rezultate poskusa na sorti 'Refošk' (*Vitis vinifera* L.), iz vinorodnega okoliša Kras (vinorodna dežela Primorska, Slovenija) v letih 2012 in 2013. V vinogradu smo izvedli tri različne obravnave: z zgodnjim razlistanjem v območju grozdja pred cvetenjem, z redčenjem grozdja v času obarvanja jagod ter kontrolo brez obravnave. Med dozorevanjem grozdja smo na izbranih trtah spremljali osnovne parametre kakovosti (pH vrednost, vsebnost suhe snovi, vsebnost skupnih titracijskih kislin), količino pridelka, listno površino in vsebnost antocianov. V primerjavi s kontrolo sta ukrepa redčenja grozdov in odstranjevanja listov pokazala trend zmanjšanja količine pridelka, zaradi zmanjšanja števila grozdov pri prvem in zmanjšanja teže grozdov pri drugem ukrepu. Pri spremljanju osnovnih kakovostnih parametrov dozorevanja nismo opazili pomembnih razlik. Je pa bila vsebnost skupnih antocianov pri obeh obravnavah v primerjavi s kontrolo višja, pri obeh obravnavah je prišlo tudi do spremembe v sestavi antocianov na račun povečanja metoksi-substituiranih monomerov. Rezultati diplomske naloge so pokazali, da se lahko vsebnost antocianov poveča tako z ukrepom redčenja grozdja kot tudi z odstranjevanjem listov pred cvetenjem.

Ključne besede: *Vitis vinifera* L. cv. 'Refošk', razlistanje pred cvetenjem, redčenje grozdja, antociani

SUMMARY

Cluster thinning and pre-flowering leaf removal are agronomical technologies used for controlling of leaf area to yield equilibrium, in order to obtain better grape quality at harvest. In this thesis the results from a trial carried out in the wine district Karst (wine-growing region Primorska, Slovenia) in the seasons 2012 and 2013 on variety 'Refošk' (*Vitis vinifera* L.) are presented. In the vineyard three different treatments were performed: control (untreated), pre-flowering leaf removal and cluster thinning at veraison. Basic quality parameteres (pH, total soluble solids, titratable acidity), yield and leaf area, and the anthocyanin content were monitored. In comparison with control, cluster thinning and leaf area removal showed a trend towards a reduction of the yield, due to cluster number reduction in the first one and cluster weight reduction in the second one. During the season no clear picture was observed regarding basic maturation parameters. However, as compared with control, in both treatments the total anthocyanin content was similar and there was also a shift in the composition, with a higher increase of methoxy-substituted monomers. The results of this study has shown that the content of anthocyanins could be increased with both cluster thinning and pre-flowering leaf removal treatments.

Key words: *Vitis vinifera* L. cv. 'Refošk', pre-flowering leaf removal, cluster thinning, anthocyanins

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ABBREVIATIONS

UNT = untreated vines

PFLR = pre-flowering leaf removal

CT = cluster thinning

Del-3-glu = delphinidin-3-glucoside

Cya-3-glu = cyanidin-3-glucoside

Pet-3-glu = petunidin-3-glucoside

Peo-3-glu = peonidin-3-glucoside

Mal-3-glu = malvidin-3-glucoside

Ac-3-glu = acetyl-glucosides

Cum-3-glu = p-coumaryl-glucosides

OMT = O-methyltransferase

F3'H = flavonoid 3'-hydroxylase

F3'5'H = flavonoid 3',5'-hydroxylase

1 INTRODUCTION

Viticulturists nowadays understand that the equilibrate ratio between leaf area and yield is crucial in order to match a good grape quality but normally the plants are unbalanced and canopy management is required to correct crop load. Normally, if the crop level is too high, clusters can be eliminated with the aim to enhance leaf area/fruit weight ratio. The same result has been shown to be achieved if leaves are removed before flowering; at this stage, leaf removal creates a stress situation for the plant that results in a lower berry-set and thus reduced cluster compactness.

Both early leaf removal and cluster thinning are known to affect also the maturation of the grapes, both in the primary and in secondary metabolism. Thus soluble solids, titratable acidity but also polyphenols and aroma compounds change in concentration as affected by cluster thinning or pre-flowering leaf removal, but in a different way. As regard polyphenols, more than just a concentration, also a change in the relative amount of single compounds can be obtained.

Since the amount of anthocyanins is particularly high for *Vitis vinifera* cv. 'Refošk', this thesis investigated how cluster thinning or pre-flowering leaf removal could lead to similar results in terms of yield reduction as compared with untreated vines, and how these techniques could affect differently a secondary metabolism of the grape berry.

1.1 Hypothesis

To improve the quality of the grapes a reduction of the yield is needed, thus the aim of the trial was:

1. To evaluate two agronomical techniques - pre-flowering leaf removal and cluster thinning – and their effects on yield parameters (berry weight, cluster weight, cluster number, yield per vine and leaf area/yield ratio) in a vineyard of *Vitis vinifera* cv. 'Refošk' grown in the slovenian Karst region;

2. To ascertain how both techniques affect the technological parameters of the grapes during maturation and at harvest (total soluble solids, pH and titratable acidity);

3. As regard anthocyanins, to understand how cluster thinning or pre-flowering leaf removal could change their biosynthesis and the relative amount of tri-substituted, di-substituted, OH-substituted and OCH_3-substituted forms.

2 THEORETICAL BASES

2.1 Leaf removal before flowering

Leaf removal, along with winter pruning, is a widespread technology around the world.

Leaf removal is known to reduce canopy density, and, as a consequence, improves canopy ventilation and light exposure of clusters (Guidoni et al., 2008; Hunter et al., 1995; Zoecklein et al., 1992). As the same time leaf removal can lead to more ripen fruit in terms of higher soluble solids and reduced acidity and can favor the accumulation of flavonols and anthocyanins (Diago et al., 2012; Hunter and Visser, 1990).

The timing when this techique is applied is very important since different results can be obtained. Usually leaf removal is performed between berry set and veraison. In the past winegrowers thought that the application around flowering should be avoided because of its negative effects on yield. Caspari et al. (1998) explained that leaf removal applied in pre-flowering time is profitable for a reduction of the berry set since less carbohydrates are available. Thus, early leaf removal used on high-yielding cultivars with large / compact clusters may achieve yield control through a reduction in fruit set and berry size and, at the same time, may lead to grape composition improvement (Poni et al., 2006; Palliotti et al. 2012). If leaf removal is performed later and/or with minimal severity, yield may not change (Hunter and Visser, 1990). On the other side, when leaf removal is excessive and late, clusters can be damaged by sunburns and/or berry color can be lowered since to high temperatures are detrimental for anthocyanin biosynthesis (Price et al., 1995).

2.2 Cluster thinning

Cluster thinning is a canopy management practice that can be used in order to limit crop level and enhance the leaf area/fruit weight ratio (Bubola et al., 2011). Other studies (Guidoni et al., 2002; Gatti et al., 2012) have shown that cluster thinning is effective on reducing yield while offering an improvement in soluble solids, berry skin anthocyanins and other flavonoids. Cluster thinning has, also, a profound effect on several important

cellular processes and metabolic pathways including carbohydrate metabolism and the synthesis and transport of secondary products. The positive effect of cluster thinning on final berry composition reflects a much more complex outcome than simply enhancing the normal ripening process (Pastore et al., 2011).

On the other side cluster thinning is an expensive agronomical tecnique, due to high labor requirements, and may not always ensure an increase in grape quality (Chapman et al., 2004; Bubola et al., 2011). In some studies (Ough and Nagaoka, 1984; Nuzzo and Matthews, 2006; King et al., 2012) cluster removal has reduced yield but has produced negative effects or had minimal effects on must sugar, pH, titratable acidity, wine color, phenolics, aroma. In the study of Keller et al. (2005), the authors wrote that cluster thinning should be considered as an auxiliary management when exceptional yield potential coincides with a cool growing season.

2.3 Polyphenols and anthocyanin profiling

Polyphenols are one of the most represented group of plant secondary metabolites and are important parameters for the quality of grapes and wine. They affect the stability and sensory qualities of the wine. They are responsible for the red color, bitterness and astringency. Polyphenols affect the perception of the body of the wine, have a stabilizing influence and are the basis for wine aging (Vanzo et al., 2012).

Total amount of anthocyanins and the relative abundance of single monomers (basic structure in figure 1) are extremely variable among red to blue-skinned cultivars (Castellarin et al., 2007). The factors that influence the profile and concentration of anthocyanins in grapes and, as a result, in wine, are: variety (main factor), terroir, vintage weather conditions (light, temperature, and the interactive effects of light and temperature), agronomic technique and winemaking (Yamane et al., 2006; Vanzo et al., 2012).

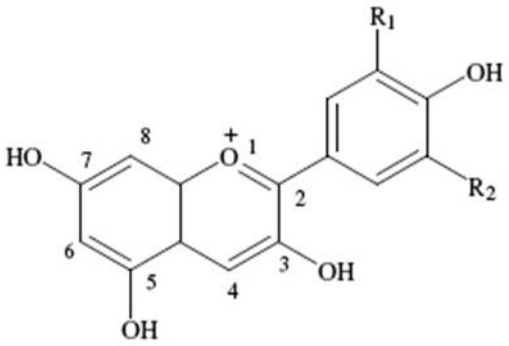

Figure 1: The basic anthocyanin structure.

As related to the substitutions at R1 and R2, different anthocyanins can be characterised as reported in Table 1.

Table 1: Disposition of the elements in anthocyanin structure (sub=substituted).

	R1	R2	di-sub	tri-sub	OH-sub	OCH_3-sub
Cya-3-glu	OH	H	X		X	
Del-3-glu	OH	OH		X	X	
Pet-3-glu	OCH_3	OH		X		X
Peo-3-glu	OCH_3	H	X			X
Mal-3-glu	OCH_3	OCH_3		X		X

Two primary anthocyanins (cya-3-glu, cyanidin-3-glucoside; del-3-glu, delphinidin-3-glucoside) are synthesized in the cytosol of berry epidermal cells. Cya-3-glu has a B-ring di-hydroxylated at the 3' and 4' positions, whereas del-3-glu has a tri-hydroxylated B-ring because of an additional hydroxyl group (OH) at the 5' position. Within the phenylpropanoid pathways (figure 2) parallel pathways of flavonoid 3'-hydroxylase (F3'H) and flavonoid 3', 5'-hydroxylase (F3'5'H) produce either cya-3-glu and del-3-glu. The 3' position of cya-3-glu and del-3-glu and sequentially the 5' position of del-3-glu can be methoxylated by OMT that generate peonidin-3-glucoside (peo-3-glu), petunidin-3-glucoside (pet-3-glu) and malvidin-3-glucoside (mal-3-glu).

Environmental stresses promote differences in the anthocyanin profiles. Castellarin et al. (2007) study showed a higher hydroxylation and methoxylation of the flavonoid B-

ring. Also sun-exposure, virus infection and other stresses can trigger change in anthocyanin profile with different relative conribution.

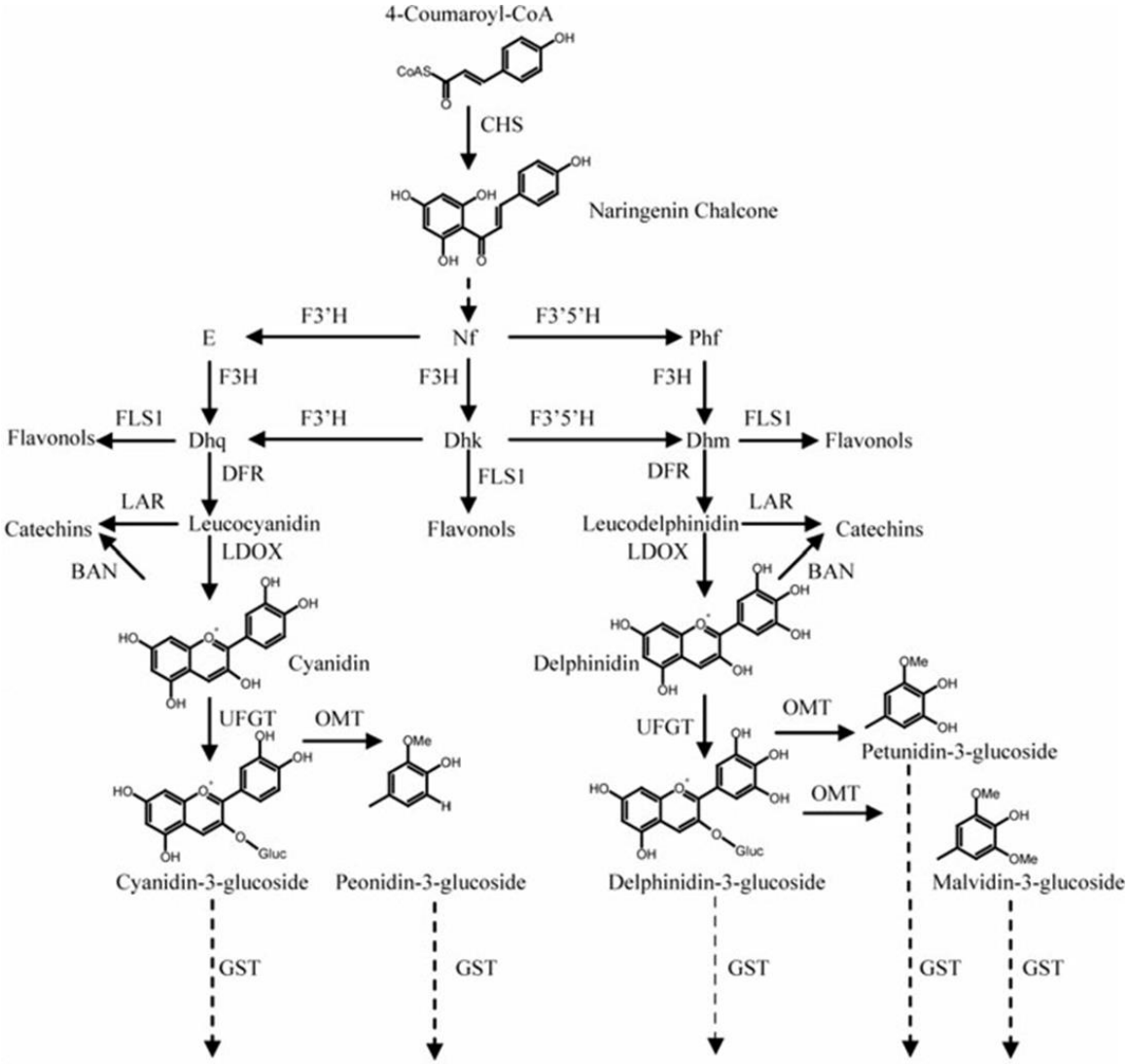

Figure 2: Anthocyanin biosynthesis (Castellarin et al., 2007).

2.4 *Vitis vinifera* L. 'Refošk'

The grapevine variety 'Refošk' is known in all four wine-region of Primorska (Slovenija), in Italian Karst and Collio and in Croatian Istria.

Adult leaves are roundish or extended; the middle part is wide; the leaf is made from three or five parts. The sinuses are shallow and tight. The upper part of the leaf is light green, in autumn becomes purple brown; along the main veins is green; the other part of the leaf is whitish and wooly; the main veins are quite prominent. The mature cluster is large or medium and thick; the peduncle is medium long, strong and green. Mature berries are medium-sized with black and purple colour. The skin is very resistant. The juice is quite acid (Mirošević and Turković, 2003).

The vine ‘Refošk' is very vigorous and abundantly born, grapes contain many polyphenols, especially those from the group of anthocyanins (Plahuta and Korošec-Koruza, 2009), but are poor in tannins (Vanzo et al., 2012).

3 MATERIALS AND METHODS

3.1 Materials

The experiment was carried out in Dutovlje, a town in the winegrowing region Primorska, within the winegrowing district Karst (figure 3). A vineyard of *Vitis vinifera* cv. 'Refosk' grafted on 420 A (*Vitis Berlandieri* x *Vitis Riparia*), with an age of 7 years was selected for the experiment. The vineyard was planted with 4444 plants x ha (0,9 m between the vines and 2,5 m between the rows), adopting a Guyot training system.

Figure 3: Location of the experimental site (Dutovlje, Karst, Slovenia).

The experiment was set randomly on selected vines with four replications for each treatment, within a split-plot experimental design as showed in figure 4 (4 replicates of 3 vines for each treatment). The replications were randomly divided into three rows.

The treatments applied on selected vines were (figure 5):

- Control – untreated vines (UNT)
- Pre-flowering leaf removal (PFLR) – performed on 25th May 2012 and 28th May 2013 - 4 to 5 leaves/shoot were removed

- Cluster thinning (CT) - performed on 2nd August 2012 and 21st August 2013 – half of the clusters were removed

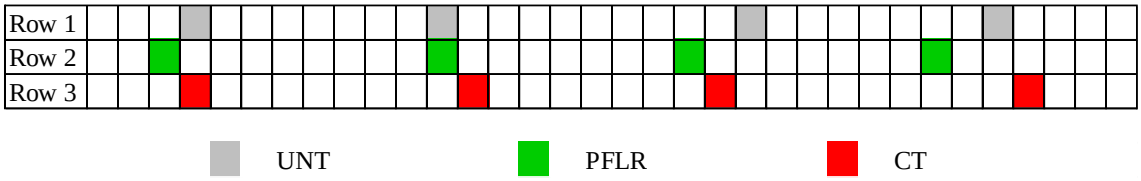

Figure 4: Trial layout in the vineyard in 2013.

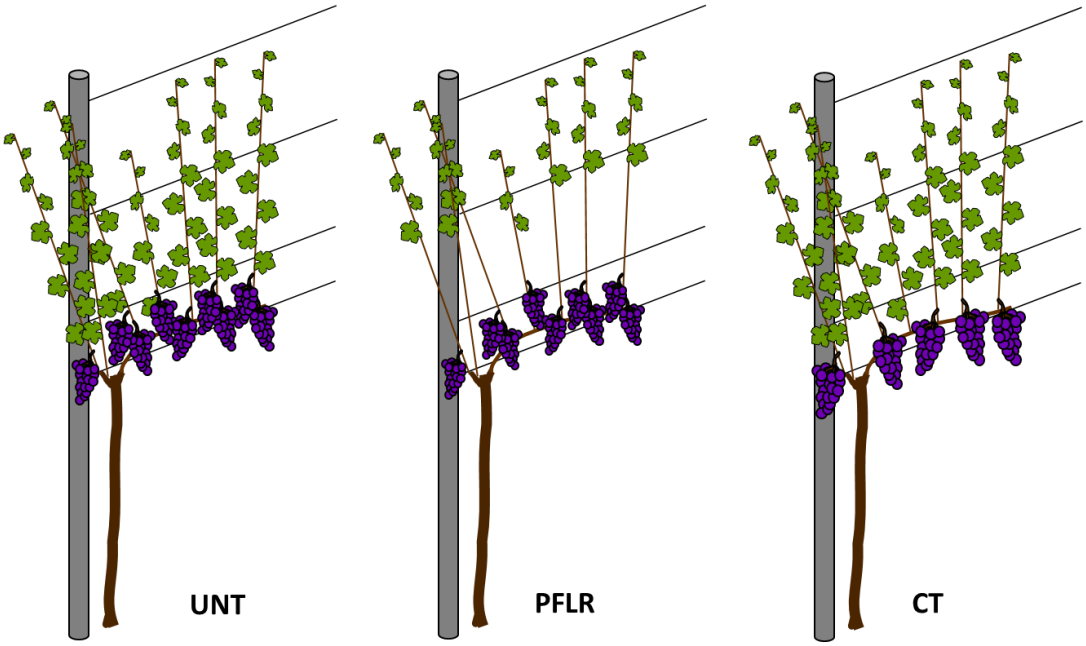

Figure 5: Scheme of the treatments (UNT, untreated; PFLR, pre-flowering leaf removal, CT, cluster thinning).

The results of 2013 were compared with the data of the previous season 2012, reported by Turk (2014) in his thesis titled "Comparison between cluster thinning and early leaf removal: effects on yield, basic quality parameters and composition of polyphenols to the variety 'Refošk' (*V. vinifera*)". The data from Turk (2014) were collected in the season 2012, except for the data about anthocyanins (the samples were frozen in 2012 and analysed in 2013), and are presented in faded colors.

3.2 Methods

3.2.1 DETERMINATION OF LEAF AREA

Leaf area was determined indirectly by using regressions between leaf and shoot parameters, keeping main and lateral leaves separated, as reported in Turk (2014).

The measurements were carried out in the vineyard, before and after pre-flowering leaf removal, and at harvest.

Two set of measurements were evaluated.

- leaf mean vein length (x) at pre-flowering time to assess single leaf area (LA) using a curvilinear regression ($LA=1{,}0537x^2 + 2{,}5597x$);
- number of leaves x shoot (n) to determine shoot leaf area (SA) using two separated regressions for main ($SA_{main}=2{,}9938n^2 + 147{,}16n$) and lateral leaves ($SA_{lat}=0{,}0594n^2 + 87{,}383n$).

The first set of measurements was performed on pre-flowering leaf removal plants to better determine the percentage of removed leaves, while the second set was adopted for all other measurements both at pre-flowering time and at harvest.

3.2.2 DETERMINATION OF YIELD, CLUSTER NUMBER, AVERAGE CLUSTER WEIGHT AND LEAF AREA/YIELD RATIO

The yield and cluster number were measured at harvest (28th September 2012 and 27th September 2013), on three plants/plot for all treatments. The yield was measured weighing the clusters per each vine. The average cluster weight was calculated as a rate between yield and cluster number. The leaf area/yield ratio, expressed in m^2/kg, was determined using the results obtained at harvest in season 2012 and season 2013.

3.2.3 DETERMINATION OF THE BERRY WEIGHT, TOTAL SOLUBLE SOLIDS, TITRATABLE ACIDITY AND pH

Portions of clusters, randomly collected from the top, the bottom and the center of the clusters, were sampled from all vines inside the same replicate. Subsamples of 100 grape berries were randomly detached and the weigh evaluated. To obtain a juice for analysis, each subsample was pressed in a plastic bag by hand, and the must separated from the pomace. For each sample of grape juice, the content of total soluble solids (°Brix), the titratable acidity (expressed as g/L of tartaric acid) and the pH value were determined. A WM-7 digital refractometer (ATAGO co. LTD., Japan) was used to ascertain the total soluble solids. The titratable acidity and the pH value were measured with a Titrino 848 automatic titrator (Metrohm, Switzerland).

3.2.4 DETERMINATION OF ANTHOCYANINS

Out of the samples collected in the field, a second subsample of 50 berries was frozen at -20°C for the later analysis of anthocyanin profile.

Each sample, still frozen, was collected from the freezer and put in a cold pool together with liquid nitrogen. Berry skins were separated carefully from the flesh and placed directly in a beaker with 100 mL of methanol for 24 hours. This procedure was carried out with liquid nitrogen in order to avoid the oxidation of the polyphenols. The extraction of phenolic compounds took place in a dark space without mixing. After 24 hours the liquid was separated, and the skins placed under a second extraction in 50 mL of methanol for another two hours (Mattivi et al., 2006). After that time the liquid was removed and the two aliquots were then combined and placed in freezer at -20˚C.

The methanol extracts were first filtered using a 0,45 μm Millipore HPLC filter. 720μL of the filtered sample were added with 80μL of 10% of trifluoracetic acid in water (TFA, Sigma, Germany) in a HPLC vial. Two technical replicates were analysed for each sample.

A Waters HPLC (Waters corporation, USA) connected with an Empower Millennium software was used for the analysis of anthocyanins with the lamp set at 520 nm.

The content of individual anthocyanins was determined by integrating the areas of individual chromatographic peaks. The chromatograms presented 5 monomeric anthocyanins (del-3-glu, cya-3-glu, pet-3-glu, peo-3-glu, mal-3-glu), 5 acetyl-glucoside and 6 coumaryl/caffeoil-glucoside derivatives. Since the recognition of the last 11 anthocyanins was not possible (missing standards), they were summed and expressed as acetyl-glucosides (ac-3-glu) and coumaryl-glucosides (cum-3-glu).

3.2.5 STATISTIC EVALUATION OF RESULTS

In order to make inference on results normally standard error is reported on graphs together with a statistics test (t-test, ANOVA). A derived index sometimes used for inference is 95% confidence interval, which is calculated from standard error. Using this statistical descriptor it is possible to claim significant differences as in Fisher LSD method (Camussi et al. 1995).

In the graphs presented in results, vertical bars thus report 95% confidence interval instead of the more used standard error or standard deviation.

The 95% confidence interval is defined as:

$$\bar{X} - 1.96\,\frac{\sigma}{\sqrt{n}} \leq \mu \leq \bar{X} + 1.96\,\frac{\sigma}{\sqrt{n}}$$

Where per each treatment, $\bar{X}$ represent the average of the data, σ the standard deviation, n the number of replicates and μ the average of the population.

4 RESULTS AND DISCUSSION

4.1 Yield, cluster weight and cluster number

The components of the yield were modified by both PFLR and CT, but in a different way as compared with the untreated vines. The plant production (figure 6) was around 3,2 and 2,5 kg/vine in the year 2012 (Turk, 2014) and 2013, respectively; a trend towards a reduction of yield was shown in the second season of the trial, probably due to the abundant rain during flowering that resulted in lower berry-set. This difference was much more pronounced in UNT than in PFLR or CT. In the first year of observation, there was a significant reduction in the yield in case of both PFLR and CT, while in the following season the yield was nearly unaffected. No differences between PFLR and CT were revealed in both seasons.

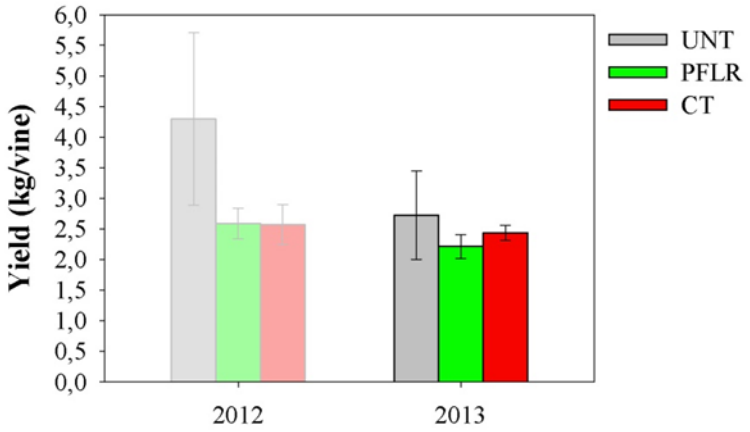

Figure 6: Effect of the treatments on the yield of 'Refošk' vines in Dutovlje (Karst, Slovenia) in 2012 and 2013 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4). Faded histograms represent data from Turk (2014).

As shown for the yield, also the average cluster weight (figure 7) was similar in the two seasons under comparison, being on average around 251g and 248g, in the year 2012 (Turk, 2014) and 2013, respectively. In both years it is possible to evaluate a trend towards a reduction in cluster weight in case of PFLR while, no differences were found

for CT treatment. With pre-flowering leaf removal, a reduction of berry-set occurs (Caspari et al., 1998) and so less berries normally account for a reduction in the average cluster weight. On the other hand, with cluster thinning normally an increase in cluster weight is obtained since the average berry weight increase and no reduction of berry-set occurs (Paladin et al., 2012).

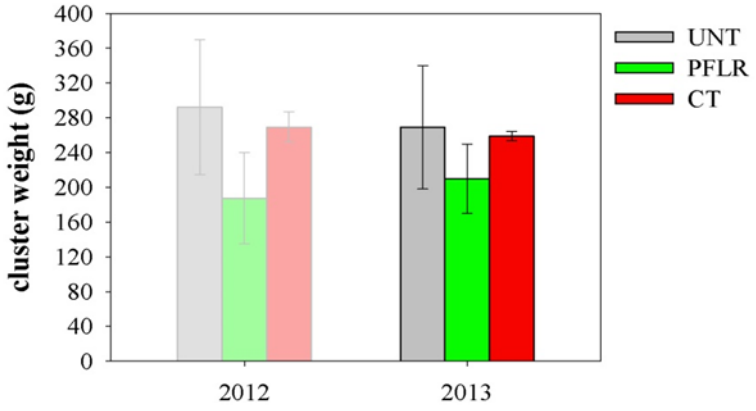

Figure 7: Effect of the treatments on the average cluster weight of 'Refošk' vines in Dutovlje (Karst, Slovenia) in 2012 and 2013 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4). Faded histograms represent data from Turk (2014).

Comparing the treatments, the only green management technique accounting for a reduction of clusters is cluster thinning, since they are physically removed from the vines. By comparing the number of clusters per vine in the two seasons (figure 8), what it is possible to show is a reduced number in the second year, being on average (before cluster thinning) 16,3 and 13,6 clusters/vine in 2012 (Turk, 2014) and 2013, respectively. As it was already discussed above, the lower number of clusters registered in the second year of trial was related with the low temperatures encountered during the first part of the season. At this stage, the completion of flower structures occurs with micro- and macrosporogenesis, and the low temperatures are known to be detrimental for the success of the last part of flower formation. Despite this fact, in case of cluster thinning, the number of initial clusters was higher and in line with the results obtained in the first season of trial 2012. Most probably was CT in year 2012 profitable for a

higher accumulation of reserves and, therefore, an increase in the bud fertility was obtained in the following season 2013. PFLR did not account for any reduction of cluster number as expected, since the clusters were not removed.

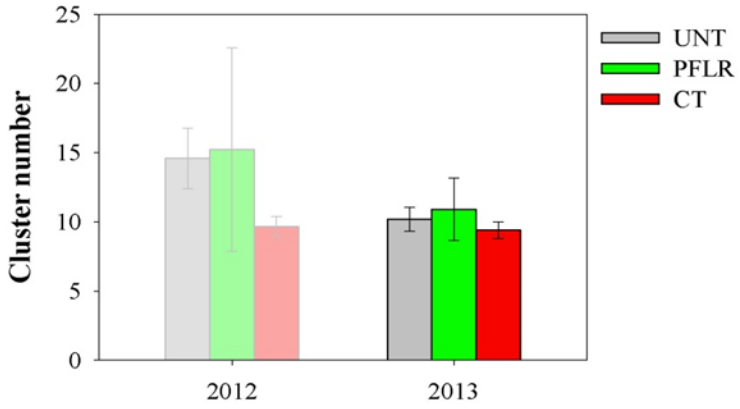

Figure 8: Effect of the treatments on the number of clusters of 'Refošk' vines in Dutovlje (Karst, Slovenia) in 2012 and 2013 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4). Faded histograms represent data from Turk (2014).

4.2 Leaf area

The leaf area of main and lateral shoots and the total leaf area (m^2/vine) before and after leaf removal was measured on 25th May 2012 (Turk, 2014) and 28th May 2013. The figure 9A, 9C and 9D show leaf area before the treatment and the figure 9B and 9E after the treatment of leaf removal. Before PFLR the different main and lateral components of leaf area were comparable between the treatments, either in 2012 than in 2013. There was a trend toward a reduced leaf area in the untreated vines in 2013, but the difference was not significant. In PFLR, the leaf area was reduced more in 2012 as compared with 2013. The leaf area on main shoots and the total leaf area in 2013 before and after removal has not been significantly reduced.

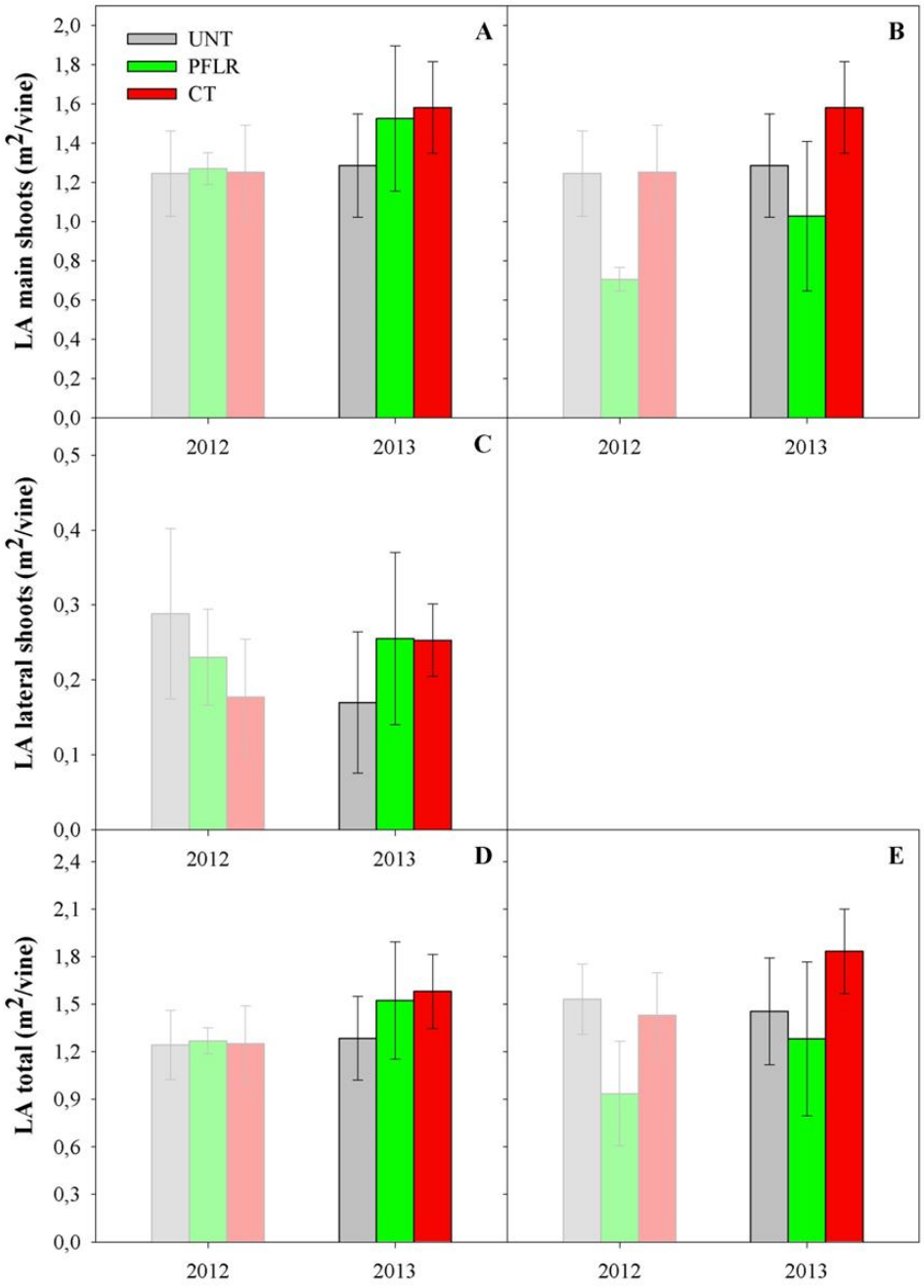

Figure 9: Leaf area (m^2/vine) on 25 May 2012 and 28 May 2013; A: leaf area (LA) of main shoots before leaf removal (LR); B: LA of main shoots after LR; C: LA of lateral shoots; D: total LA before LR; D: total LA after LR (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4). Faded histograms represent data from Turk (2014).

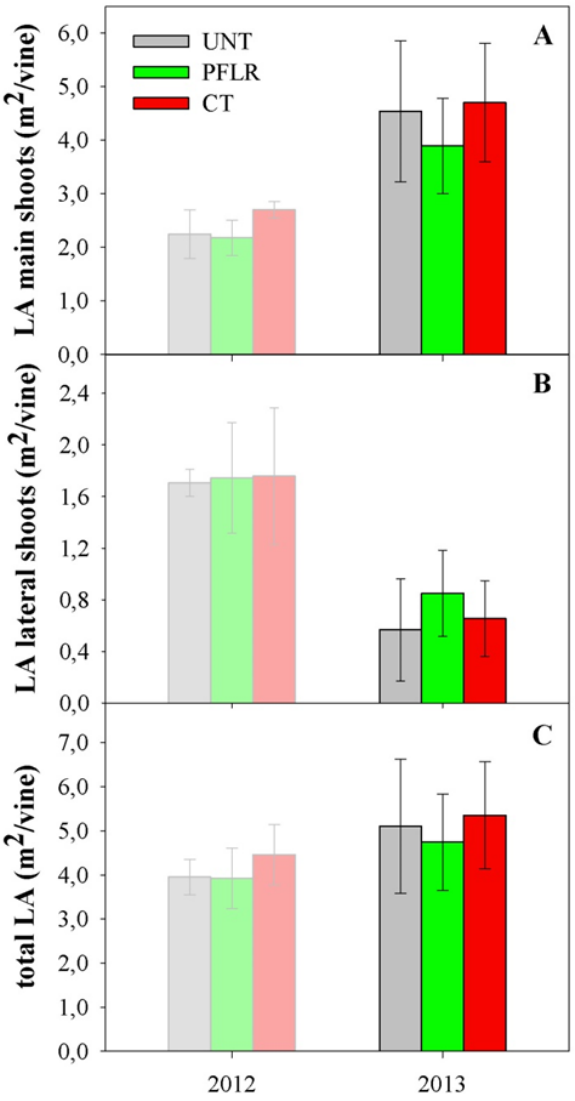

Figure 10: Leaf area (m^2/vine) on 28 Sept 2012 and 27 Sept 2013; A: leaf area (LA) of main shoots; B: LA of lateral shoots; C: total LA (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4). Faded histograms represent data from Turk (2014).

The leaf area at harvest was measured on 28th September 2012 (Turk, 2014) and 27th September 2013 (figure 10). Comparing the seasons 2012 and 2013, there are substantial differences in the leaf area values of main and lateral shoots, but without any

statistical differences in total leaf area at harvest. The leaf area was the same for all treatments in each year, either in 2012 than in 2013. As regard PFLR, many authors showed an increase of lateral leaf area (Diago et al., 2012; Poni et al., 2006) as compared with untreated vines, and even if not significant, a trend towards an increase of lateral leaf area was revealed in our experiment in the season 2013.

4.2.1 LEAF AREA/YIELD RATIO

Kliewer and Dokoozlian (2005) showed that an equilibrate ratio between leaf area and yield should be between 0,7-1,4 m^2/kg in order to obtain a good maturation of the grapes. In the present experiment, leaf area/yield ratio was shown to be optimal or higher than optimal, mainly for CT and PFLR. In the year 2013, there was a trend toward a higher rate as compared with 2012, expecially for PFLR (figure 11). However, there were not statistical differences between the treatments in both years, mainly because of the high variability of leaf area (figure 9).

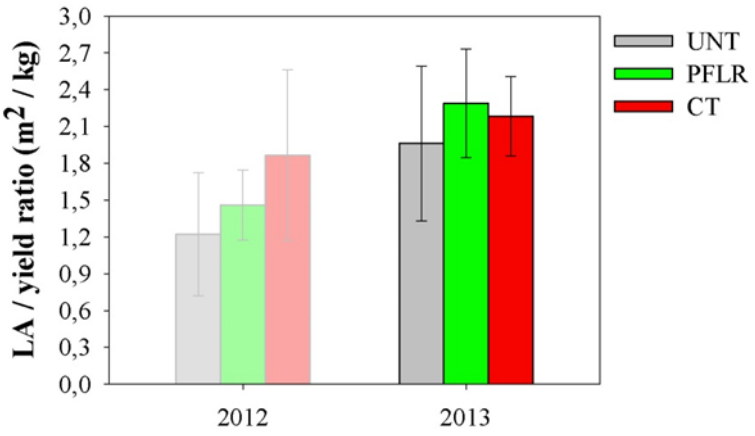

Figure 11: The ratio between leaf area and yield in 2012 and 2013 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4). Faded histograms represent data from Turk (2014).

4.3 Berry weight, total soluble solids, titratable acidity and pH

Berry weight did not show significant differences between treatments during maturation (figure 12A). However, there was a trend toward a lower berry weight in CT during maturation, with PFLR being intermediate. The average weight was similar in 2012 and 2013, with slightly higher values in 2013 (figure 12B). Bravdo et al. (1985), Gil et al. (2013) and Paladin et al. (2012) found an increase of berry weight with cluster thinning, while Guidoni et al. (2008) in their experiments revealed no differences with unthinned grapevines. Di Profio et al. (2011), working on different varieties, found completely different behaviour in Merlot (reduction of berry weight), Cabernet Sauvignon (increase) and Cabernet franc (no differences). Thus the effect of cluster thinning can be dramatically different as relate also with the meteorological course of the season.

As regard PFLR, a trend towards a reduction was shown in both seasons as compared with the control. Same results were showed also by Poni et al. (2006), in Sangiovese and Trebbiano, and by Sternad Lemut et al. (2011) in Pinot noir, while Lee and Skinkis (2013) found no differences comparing PFLR and UNT.

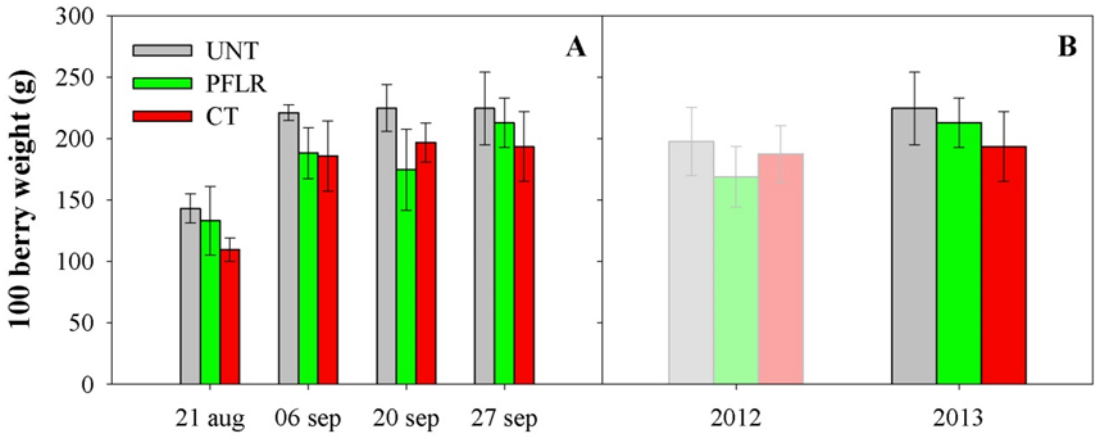

Figure 12: Modification of 100 berry weight during maturation in 2013 (A) and compared with 2012 (B). (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4). Faded histograms represent data from Turk (2014).

During maturation, the content of total soluble solids (°Brix) was the same in all treatments (figure 13A), except for the harvest date (27th Sept 2013), when a slightly lower value was shown in case of CT. In the previous season 2012 (figure 13B), on the contrary, there was an increase in total soluble solids content in CT. PFLR did not emphasised any particular increase/reduction as compared with UNT.

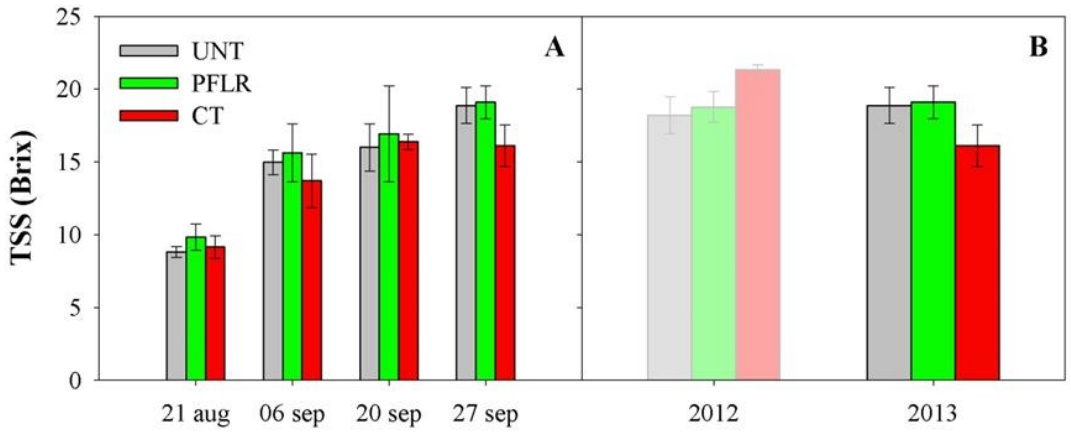

Figure 13: Modification of total soluble solids content during maturation in 2013 (A) and compared with 2012 (B). ***(UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4). Faded histograms represent data from Turk (2014).***

The behaviour of CT in the season 2013 is somehow "particular" as compared with 2012. What we would have expected, it was the same trend, since normally there is an inverse relationship between yield and sugar accumulation in berries (Kliewer and Dokoozlian, 2005). There are anyway other trials explaining different behaviour between CT and UNT also between seasons. Paladin et al. (2012) showed increase or reduction of sugars in CT vines in the same season and for the same variety. On the other hand, Kliewer and Weaver (1971), Reynolds et al. (2007), Di Profio et al. (2011), and Gil et al. (2013), showed similarly an increase of soluble solids in the treatments where cluster thinning was applied.

As regarding PFLR, there are some reports that highlight no differences with UNT (Sternad Lemut et al., 2011; Lee and Skinkis, 2013; Risco et al., 2014) or increased soluble solids in Sangiovese and Trebbiano (Poni et al., 2006), in Pinot gris (Sternad Lemut et al., 2011) and Tempranillo (Diago et al., 2012).

The content of titratable acidity in 2013 (figure 14A) was the same between treatments, but there was a reduction during maturation. In 2013 the level of titratable acidity was higher than in 2012, mainly in case of CT (figure 14B).

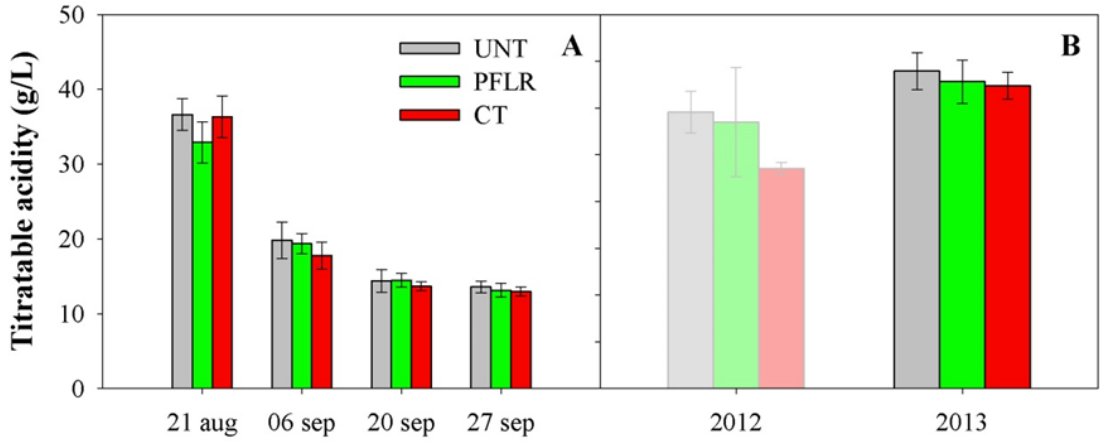

Figure 14: Modification of titratable acidity during maturation in 2013 (A) and compared with 2012 (B). *(UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4)*. Faded histograms represent data from Turk (2014).

In other experiments carried out in different varieties, the authors basically observed a trend towards a reduction in titratable acidity (Di Profio et al., 2011; Paladin et al., 2012; Gil et al., 2013) comparing CT with UNT.

As regard PFLR, no differences of titratable acidity were found by Lee and Skinkis (2013) in Pinot noir, by Poni et al. (2006) in Trebbiano and by Risco et al. (2014) in Tempranillo. As opposite, Poni et al. (2006) found an increase of titratable acidity in Sangiovese and Diago et al. (2012) a reduction of the same parameter in Tempranillo.

The trend of pH evolution during maturation 2013 can be observed in the figure 15A, which remained similar between treatments, except for CT in the last date, that was lower than PFLR and UNT. In the season 2013 the average values of pH were lower than in the previous season 2012 (figure 15B).

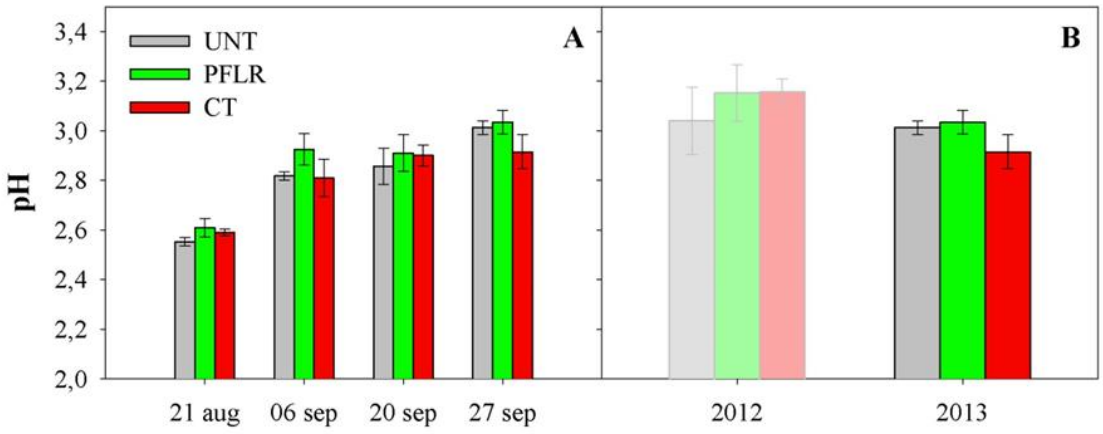

Figure 15: Modification of pH during maturation in 2013 (A) and compared with 2012 (B). ***(UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4). Faded histograms represent data from Turk (2014).***

Other researchers found an increase of pH in case of CT (Di Profio et al., 2011; Gil et al., 2013). On the other hand for PFLR the results showed in other experiments revealed no appreciable effects on this parameter (Poni et al. (2006) on Sangiovese and Trebbiano; Risco et al. (2014) on Tempranillo).

4.4 Total anthocyanins content

The content of total anthocyanins (figure 16) in 2012 was higher in PFLR and CT as compared with UNT, but substantially similar between them. In 2013 anthocyanin content was higher and there was a huge variability between replicates, thus no

differences were revealed between treatments. Anyway a trend towards a higher concentration in CT and PFLR treatments was shown.

Di Profio et al. (2011) working on Merlot, Cabernet Franc and Cabernet Sauvignon, Gatti et al. (2012) on Sangiovese, and Sternad Lemut et al. (2011) on Pinot noir found an increase in total anthocyanins content with cluster thinning, while Gil et al. (2013) on Syrah registered a decrease of the same parameter. Paladin et al. (2012) found either increase or reduction of total anthocyanins in different vineyards where cluster thinning was performed. On the other hand, Sivilotti and Lavrenčič (2010) revealed an increase of anthocyanins with 50% of cluster thinning, however an opposite trend was observed when 75% of the same treatment was applied.

As regard pre-flowering leaf removal, many authors showed an increase in total anthocyanins content on different varieties (Poni et al., 2006; Diago et al., 2012; Gatti et al., 2012; Matsuyama et al., 2014), while Risco et al. (2014) did not found differences in Tempranillo as compared with untreated vines.

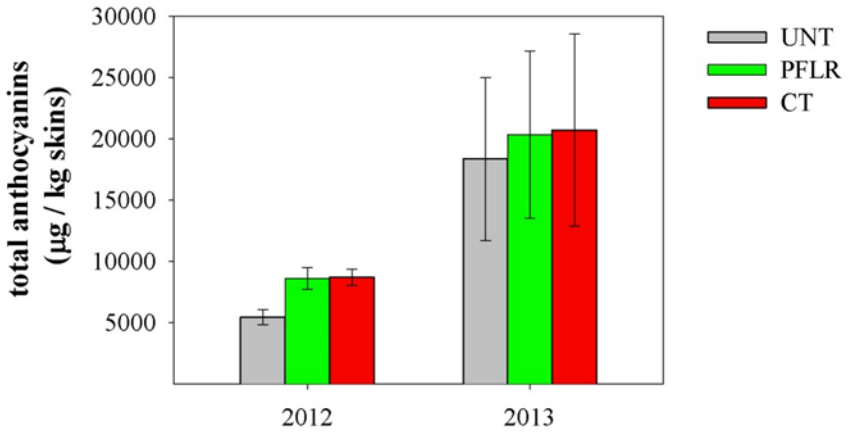

Figure 16: The content of total anthocyanins in mg/kg of skins in 2012 and 2013 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4).

The content of del-3-glu, cya-3-glu, pet-3-glu, peo-3-glu, mal-3-glu, ac-3-glu and cum-3-glu in seasons 2012 and 2013 is shown on figure 17 and figure 18, respectively. In the

first season 2012, the highest contents of single anthocyanins were revealed in case of CT with the exception of mal-3-glu and acetylated/p-coumarylated forms, that were higher in PFLR. However, the content of all anthocyanins was as a trend higher in CT and PFLR as compared with the UNT grapes. In the season 2013, probably because of the higher content of anthocyanins and the wide variability between replicates, no differences were found. Anyway a trend towards a higher content in PFLR and CT treatments was revealed.

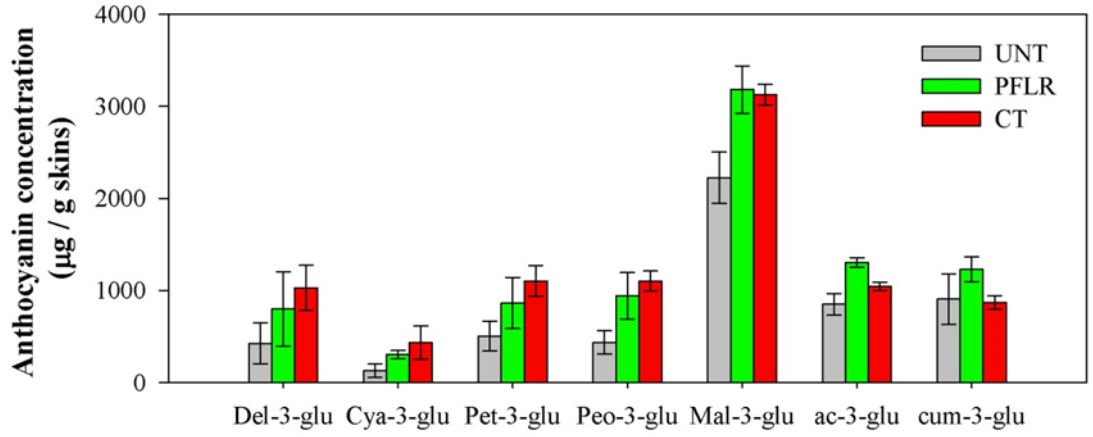

Figure 17: Content of del-3-glu, cya-3-glu, pet-3-glu, peo-3-glu, mal-3-glu, ac-3-glu and cum-3-glu in 2012 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4).

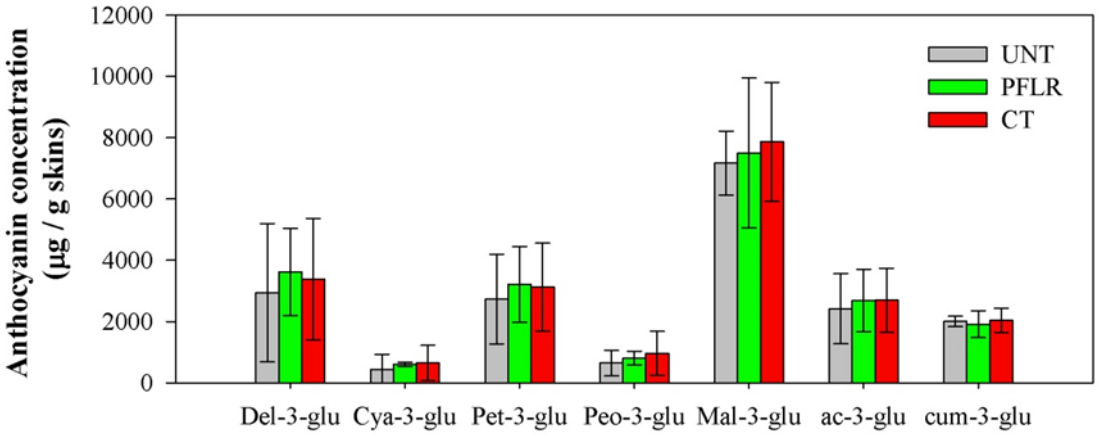

Figure 18: Content of del-3-glu, cya-3-glu, pet-3-glu, peo-3-glu, mal-3-glu, ac-3-glu and cum-3-glu in 2013 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4).

The concentration of mal-3-glu was significantly higher than others anthocyanins in both seasons, but in 2013 the amount was nearly double than in 2012. In the season 2013 the content of tri-substituted anthocyanins (del-3-glu, pet-3-glu and mal-3-glu) (figure 19) was significantly higher than in 2012, while the concentration of di-substituted anthocyanins (cya-3-glu and peo-3-glu) remained nearly the same (figure 20).

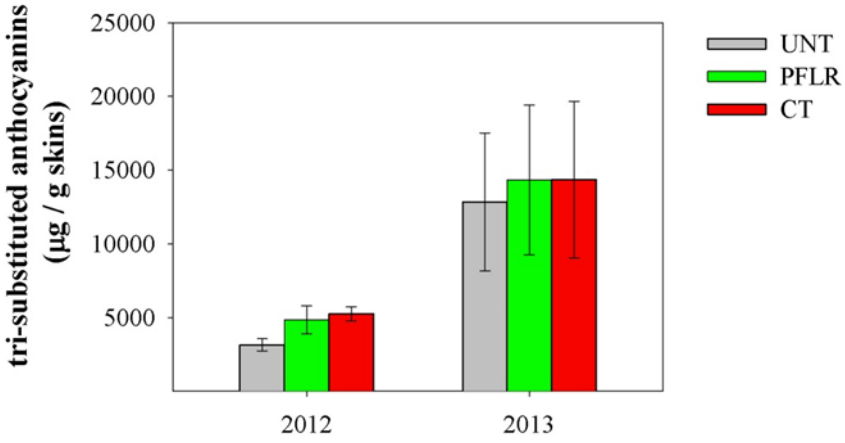

Figure 19: Content of tri-substituted anthocyanins in 2012 and 2013 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4).

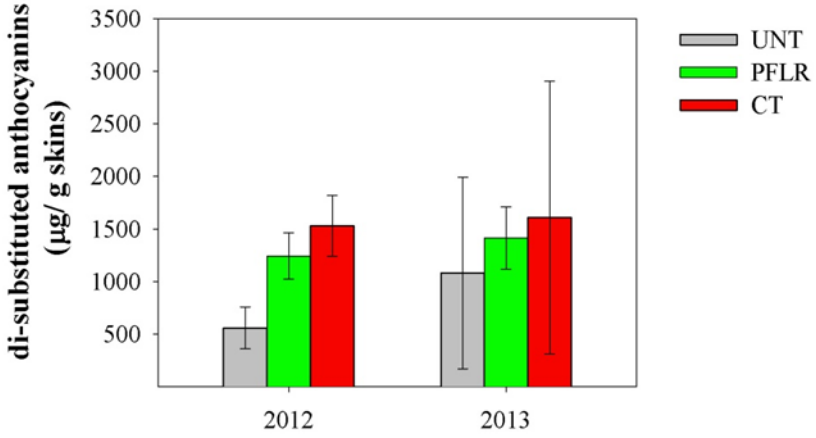

Figure 20: Content of di-substituted anthocyanins in 2012 and 2013 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4).

The content of tri-substituted anthocyanins was higher in PFLR and CT as compared with UNT. On the other hand in the following season 2013, even if there is a trend similar to 2012, no significant differences were revealed among treatments.

As regard the di-substituted anthocyanins in 2012 statistically lower values were shown in untreated vines, while in CT and PFLR the concentration was approximately the same. In 2013, less differences among treatments were shown, but still a trend towards a higher content in PFLR and CT could be appreciated.

Castellarin et al. (2007) in their study found than grapevine subjected to water or other biotic stresses increased the content of tri-substituted anthocyanins as compared with the di-substituted ones that remained unaltered. Matsuyama et al. (2014) in one experiment carried out on *Vitis labruscana x V. Vinifera* cv. 'Muscat Bailey A' subjected to leaf removal, showed a higher increase of tri-substituted anthocyanins in this treatment as compared with untreated vines.

While the concentration of anthocyanins increase, the expression of targeted genes coding for phenylpropanoids is supposed to be enhanced (Castellarin et al., 2007). Among genes, F3'H, F3'5'H and OMT were shown to change as affected by biotic stresses (drought, sun exposure, etc.). If F3'5'H expression increases, thus tri-substituted anthocyanins are promoted more than di-substituted ones. When OMT expression increases, higher concentration of pet-3-glu, mal-3-glu and peo-3-glu will be revealed.

In the season 2013 the content of OH-substituted anthocyanins (cya-3-glu and del-3-glu) (figure 21) as a trend was higher than in 2012, and in each year differences among treatments were shown. Even if not significant, in both PFLR and CT a trend towards higher concentration of OH-substituted anthocyanins could be observed.

As opposite, the concentration of OCH_3-substituted anthocyanins (pet-3-glu, peo-3-glu and mal-3-glu) (figure 22) was significantly higher in 2013 as compared with 2012. In the first season 2012, both PFLR and CT accounted for significant higher content of OCH_3-substituted anthocyanins as compared with UNT, while in the following season 2013 no differences among treatments were shown.

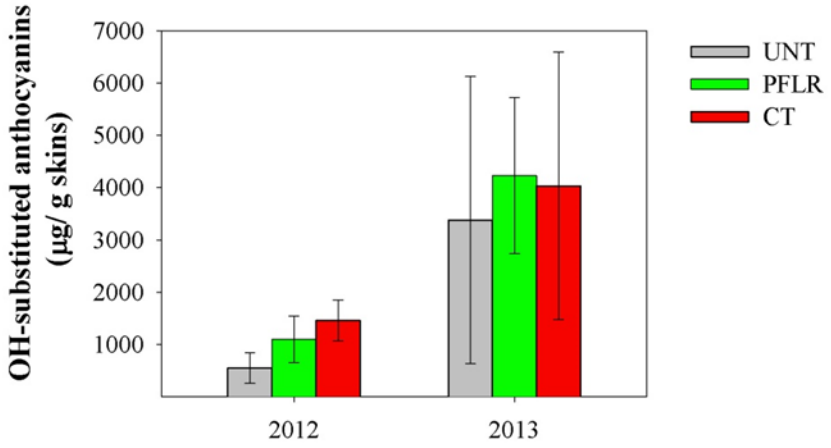

Figure 21: Content of OH-substituted anthocyanins in 2012 and 2013 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4).

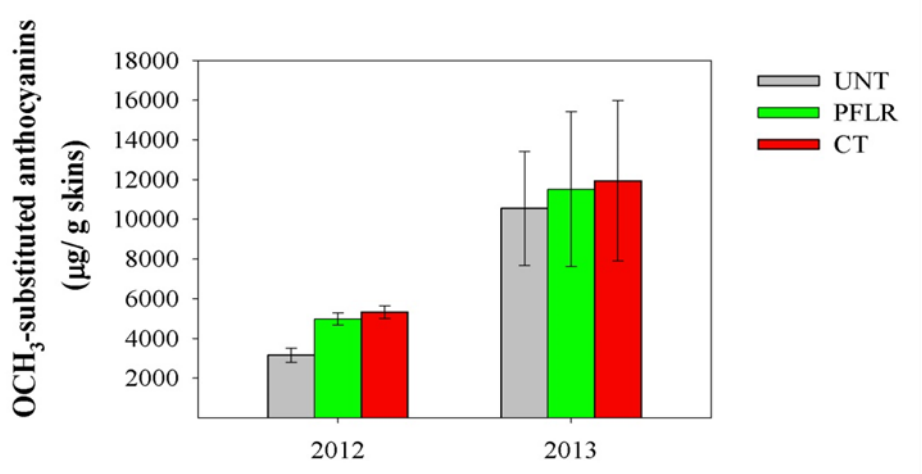

Figure 22: Content of OCH_3-substituted anthocyanins in 2012 and 2013 (UNT – untreated, PFLR – pre-flowering leaf removal, CT – cluster thinning) (bars represent 95% confidence interval, n=4).

5 CONCLUSIONS

The overview of the results is presented in three points, as they were listed at the beginning of the thesis under the chapter “Hypothesis”.

1. Effects of pre-flowering leaf removal and cluster thinning on yield parameters

In the season 2013 yield per vine was similar in PFLR and CT and lower than in UNT. There was a trend toward a reduction in the average cluster weight in case of PFLR as compared with both UNT and CT, while no differences between UNT and CT were found. On the other hand, berry weight was not significantly affected by treatments. A trend towards an increase of leaf area / yield ratio was shown for both cluster thinning and early leaf removal, over the optimal range of 1,0-1,4 m^2/kg proposed by Kliewer and Dokoozlian (2005). For 'Refošk' an optimal equilibrium was already shown for UNT, thus we could speculate that a further increase could be not always profitable for a better grape quality.

2. Effects of pre-flowering leaf removal and cluster thinning on technological parameters during maturation and at harvest

In the season 2013 the total soluble solids increased in concentration during maturation, with trendly lower values in case of CT and similar values for UNT and PFLR.

During maturation, titratable acidity was reduced with no differences between treatments.

Significant differences in pH value were reported for CT with lower pH values at harvest as compared with PFLR and UNT.

3. Effects of pre-flowering leaf removal and cluster thinning on anthocyanins

The concentration of total anthocyanins in the two vintages was very different. In the season 2013 the content was more than double as in 2012. As regard treatments, higher

contents were shown for PFLR and CT in the season 2012, while no differences were revealed in 2013. No significant differences were shown between PFLR and CT at harvest 2012 as compared with UNT.

Even if not all anthocyanins showed significant differences among treatments, the content of del-3-glu, cya-3-glu, pet-3-glu and peo-3-glu was higher in CT in the season 2012, while the content of mal-3-glu was higher and similar in CT and PFLR, and acetyl- and p-coumaril-derivatives were higher in PFLR. The content of del-3-glu, pet-3-glu, mal-3-glu and acetyl- and p-coumaril-derivatives was increased in the second season 2013, while the content of cya-3-glu and peo-3-glu remained about the same. However, because of the high variability of the replicates, no differences between treatments were shown in the season 2013.

Tri-substituted anthocyanins were higher in the season 2013, with similar values between treatments, while in 2012 a higher content was found for PFLR and CT as compared to control. On the other hand, the content of di-substituted anthocyanins was similar between seasons and treatments, with lower values only for UNT in 2012.

Less differences among treatments were found for both OH-substituted and OCH_3-substituted anthocyanins, while differences between seasons where again shown.

In summary, leaf removal before flowering and cluster thinning revealed to be both important in triggering changings in the content and profiling of anthocyanins in grapes with somehow comparable results between them.

6 LITERATURE

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