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Girdling stimulates anthocyanin accumulation and promotes sugar, organic acid, amino acid level and antioxidant activity in red plum: an overview of skin and pulp metabolomics

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Abstract

Girdling is a widespread agronomic technique to increase the fruit quality characteristics (e.g. size, solid soluble content [SSC] and colour). However, the information on the fruit metabolic

changes related to this practice still remains unclear and fragmentary. Moreover, girdling duration and application time may greatly affect the plant/fruit metabolic responses producing sometimes counterproductive results. Fruit quality, metabolomic and antioxidant analyses were conducted to characterise the effects of two different girdling dates (4- and 2-weeks before the harvest, 4W and 2W, respectively) in skin and pulp of red-fleshed plum (*Prunus cerasifera* var. *pissardii*). Overall, the pulp metabolism was altered in both 4W and 2W Girdling by inducing accumulation of sugars (sucrose and, trehalose), sugar alcohols (inositol and xylitol), organic acids (especially some TCA cycle intermediates such as α-ketoglutaric, citric, isocitric, fumaric and malic acid), amino acids (ß-Alanine and L-Proline), anthocyanins and other phenols., whereas inIn the skin only girdling 4W showed major significant differences compared to the control increasing the fruit quality characteristics (size, SSC, dry matter and red colour) and showing greater metabolic changes with respect to the controls. Furthermore, the total antioxidant activity was also increased in both skin and pulp respect to other treatment only in Girdling 4W. This approach could be used with both *P. cerasifera* plums as well as other red-fleshed fruit species in order to ensure red-fleshed fruits production with a uniform red colouration and higher content of bioactive compounds.

Keywords: anthocyanin, colour, fruit, metabolomics, organic acid, sugar

1. Introduction

Nowadays, there is a growing demand from consumers for novel and bioactive-enriched fruits, in particular for red/purple fruits which include most of major antioxidant food sources (Espley et al., 2013; Hidalgo and Almajano, 2017; Khoo et al., 2017; Gramza-Michałowska et al., 2019). The fruit colouration in the pulp and especially in the skin (attractive qualities for consumers) is attributable to the presence of pigments i.e. chlorophylls, carotenoids and anthocyanins (Willson and Whelan, 1990; Kayesh et al., 2013).

Through the years, different agronomical strategies have been tested in order to improve fruit qualities (e.g. size, colour, soluble solid content, dry matter), and among these, the girdling technique have provided interesting results. Girdling is an old worldwide horticultural practice applied to increase flowering, fruit set and fruit size, maturity and higher quality, and to alter wood properties in forestry (Gawankar et al., 2019). Girdling is a surgically-induced stress which consists in removal of a ring of bark leading to an accumulation of carbohydrates above the girdling area (Goren et al., 2010). This technique is commonly used in grape production for enhancing berry colour and size, maturation and soluble solid content (SSC) (Koshita, 2015; Basile et al., 2018).

Girdling treatment, performed at different fruit developmental stages, was also tested on large branches of fruit trees for enhancing fruit attributes and it was found that fruit colour and weight were improved in some stone fruits belonging to the *Prunus* ~~family~~ genus (Agusti et al., 1998). In a 2-year study conducted in Idaho, the girdling treatment was tested on 'Aztec Fuji' apple trees and it was shown to increase during the first year the fruit size, colour and firmness (Fallahi et al., 2018). Other positive effects on fruit quality given by girdling were found on barberry (*Berberis vulgaris*), kiwifruit (*Actinidia chinensis*) and sweet cherry (*Prunus avium*) (Nardozza et al., 2015; Mertes et al., 2016; Michailidis et al., 2020).

The reddish colouration in fruits mainly depends on the biosynthesis of colourful flavonoids, namely anthocyanins (Willson and Whelan, 1990; Kayesh et al., 2013). Noel (1970) hypothesised that, through phloem flux interruption, with a consequent sugar accumulation, anthocyanin biosynthesis could be positively affected, thereby leading to an increase in red colouration in plant tissues. Nowadays, some researchers have found that many of the genes encoding enzymes involved in anthocyanin biosynthesis are largely regulated by environmental factors such as light, temperature UV wavebands and also a clear correlation between sugar accumulation and anthocyanin production has been proven (Solfanelli et al., 2006; Das et al., 2012; Lo Piccolo et al., 2018; Nardozza et al., 2019; Lo Piccolo et al., 2020b).

Few works have evaluated the metabolic changes due to girdling treatment in fruits (Yang et al., 2013; Basile et al., 2018; Michailidis et al., 2020). It is conceivable that an increase in girdling-promoted sugar content in fruit tissue leads to increased biosynthesis of other molecules such as amino acids, vitamins, phenols and other bioactive compounds which are useful also for human health (Yang et al., 2013; Michailidis et al., 2020). Moreover, many works analysed the effect of girdling at the early-growing season during the fruit set stage (Agusti et al., 1998; Gawankar et al., 2019; Michailidis et al., 2020). On the contrary, less information is available about late season girdling effects (Basile et al., 2018; Day and DeJong, 1998), raising doubts about the consistency of fruit growth promoting effects by this technique when applied at late stages (Day and DeJong, 1998). Besides, it still remains to elucidate whether late girdling treatment affects the fruit physiological responses, and the treatment duration leads to different results.

The production of fruits with enriched antioxidant proprieties could further satisfy the increasing demand of 'nutrafruit' (fruits with good nutritional/nutraceutical characteristics), for its positive correlation with human health (Szajdek and Borowska, 2008; Battino et al., 2009).

Prunus cerasifera Ehrh., commonly called myrobalan or cherry plum, belonging to the *Rosaceae* family, is native to the Southeastern Europe to western Asia. The *P. cerasifera* var. *pissardii* has been widely used as ornamental tree species, even though its reddish edible fruits have also been studied for their richness in antioxidants and vitamins (Kırbağ and Göztok, 2016). Previous works, conducted on leaves of *P. cerasifera* var. *pissardii*, highlighted a close correlation between leaf carbohydrate content and anthocyanin accumulation (Lo Piccolo et al., 2018). A rapid change of leaf colour toward a darker red (supportive for higher anthocyanin level) was observed following accumulation of soluble sugars either in case of sink-source manipulation (Lo Piccolo et al., 2020b) or in natural conditions (Lo Piccolo et al., 2018, 2020a).

In view of the strict interplay between sugar metabolism, anthocyanin accumulation and antioxidant properties of the whole fruit, we hypothesized that the effect of bark girdling influences *P. cerasifera* fruit features. In particular, we tested whether fruits of *P. cerasifera* var. *pissardii* were influenced by two different girdling treatment, performed at late fruit growth stage (4- and 2-weeks before the harvest), in both physio-quality attributes (size, colour, SSC) and/or metabolomics profile at both pulp and skin level. To elucidate the possible time-effect of late girdling ('final sweel' stage) in *P. cerasifera* var. *pissardii* fruits, girdling treatments were applied and a deep untargeted metabolomics approach was used to depict the fruit biochemical features after girdling.

2. Materials and Methods

2.1. Plant material

Experiments were carried out with fruits collected from five 10-year-old *Prunus cerasifera* var. *pissardii* trees, grown in the garden of the Department of Agriculture, Food and Environment of the University of Pisa (43°42′40.9″N 10°24′40.9″E). Girdling treatment was applied at two experimental time points (4- and 2- weeks, 4W and 2W respectively) before the

harvest time (June). For the experiments on plants, two homogeneous (in terms of canopy position, light exposition, dimension and leaf number) 2-year-old branches were chosen for each treatment in each tree. Girdling treatment was performed at 1.5 m from the end of the tree branch (⌀ 15 mm).

On June, at harvest time, 20 plum fruits were sampled (2 per branch per each tree) for each treatment. In all the experiments, singly-grown fruits were harvested at 11 a.m. Fruit were washed twice with tap water and finally with distilled water before their peeling. Physical and chemical harvest indexes were estimated for each sampled fruit and included: (i) fresh weight (FW; g), height (mm), width (mm), (ii) skin and pulp dry matter (%) and ~~soluble solid content~~SSC (~~SSC, °~~Brix), which was determined in flesh juice samples using a digital refractometer (Mod. 53 011, Turoni, Forli, Italy), (iii) skin and pulp colour. Dry matter content of pulp and skin samples was determined gravimetrically by oven drying at 65 °C until constant weight (n = 20). The remained pulp and peel sample parts were cut into small pieces (about 5 x 5 x 5 mm), placed in Falcon tubes, frozen in liquid nitrogen, and stored at -80 °C for biochemical analyses.

2.2. Colour analysis

Fruit skin and pulp colour (n = 20) was recorded using standard CIELab, color space coordinates determined using a spectrometer Ocean Optic HR2000-UV–VIS-NIR (Ocean Optics, Florida, USA). Values of lightness (L*), redness and greenness (a* and –a*), yellowness and blueness (b* and –b*) were determined on the hue circle. Chroma (C*) was calculated as $(a^{*2} + b^{*2})^{1/2}$, and the hue angle, hue = arctg (b*/a*), expresses the colour nuance.

2.3. Pigment analyses

Total anthocyanin concentration (TA) was determined using the pH differential method (Giusti and Wrolstad, 2001). About 100 mg of fresh skin and pulp material was extracted in acidified methanol (1.5% HCl, v/v) and kept overnight at room temperature. The absorbance was recorded at 530 and 700 nm using an Ultrospec 2100 Pro UV–VIS spectrophotometer (GE Healthcare Ltd., Chicago, IL). The final absorbance (A_f) of diluted samples was calculated as follows:

$$A_f = (A_{530} - A_{700})_{\text{pH 1.0}} - (A_{530} - A_{700})_{\text{pH 4.5}}$$

TA was expressed as cyanidin-3-*O*- glucoside (molar extinction coefficient of 34,300 L cm^{-1} mol^{-1} and molecular weight 484.3 g mol^{-1}) equivalents. Measurements of TA was determined in each analysed fruit sample (n = 20).

Total chlorophyll (Chl_{TOT}) and carotenoid (Car) concentrations were determined according to Zhang and Kirkham (1996) with some modifications. The analyses were conducted in five replicates, using 4 fruit samples per replicate (n = 20). About 50 mg of freeze-dried fruit samples (skin and pulp), were ground in 10 mL of 80% aqueous acetone. The homogenate was centrifuged at 7,000 *g* for 10 min. The supernatant was collected and the pellet was suspended in 1 mL of 80% aqueous acetone and centrifuged again as mentioned above. Elution and centrifugations were repeated until the pellet was completely discoloured. The supernatant absorbances at 663, 648 and 470 nm were measured. Chl_{TOT} were measured as the sum of Chl *a* and *b.* Chl *a* and *b* content and total carotenoid concentration were calculated according to Lichtenthaler (1987).

2.4. Total Antioxidant Activity (TAA) analysis

Freeze-dried skin and pulp samples (about 0.05 g FW) were homogenised with 1 mL of 70% (v/v) 99.5% HPLC grade methanol by sonication for 30 min, keeping the temperature

within the range 0 to 4 °C. After centrifugation (6,000 *g* for 10 min at 4 °C), supernatants were collected and filtered through PTFE filters (0.20 µm pore size; Sarstedt, Verona, Italy). The extractions were conducted in five replicates, using 4 fruits per replicate (20 fruits in total). Extracts were stored at −80 °C before analysis. To estimate the TAA, the 2,2-difenil-1-picrylhydrazyl (DPPH) assay was done according to Brand-Williams et al. (1995) method. Briefly, 15 µL of previous extract was added to 2.985 mL of a solution containing 3.12×10^{-5} M DPPH in methanol. The decrease in absorbance at 515 nm was measured against a blank solution (without extract) after 30 min of reaction time at room temperature (optimised for the highest antioxidant concentrations in the extract) using a spectrophotometer (Ultrospec 2100 Pro UV-VIS). Results are expressed as mg Trolox equivalent (TE) g^{-1} FW.

2.5. Metabolome extraction, derivatisation, and GC-MS analysis

Plant materials were collected and immediately snap frozen in liquid nitrogen to quench the endogenous metabolism. Freshly homogenisz~~z~~ed (100 mg) plant material, for each sample and replicates, was lyophilisz~~z~~ed at -40 °C. Extraction, internal standard addition (ribitol 0.2 mg ml^{-1}) and dativisz~~z~~ation was carried out following the protocol described by ~~(~~Lisec et al.~~,~~ (2006).

The derivatised extracts were injected into a MEGA-5MS capillary column (30 m x 0.25 mm x 0.25 µm + 10 m of pre-column) (MEGA S.r.l., Milan, Italy) using a gas chromatograph apparatus (Trace GC 1310, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a single quadrupole mass spectrometer (ISQ LT, Thermo Fisher Scientific, Waltham, Massachusetts, US). Injector and source were set at 250 °C and 260 °C temperature, respectively. One µl of sample was injected in splitless mode with a helium flow of 1 ml min^{-1} using the following programmed temperature: isothermal 5 min at 70 °C followed by a 10 °C min^{-1} ramp to 350 °C and a final 5 min heating at 330 °C. Mass spectra were recorded in electronic impact (EI) mode at 70 eV, scanning at 40-600 m z^{-1} range, scan time 0.2 sec. Mass

spectrometric solvent delay was settled at 7 min. Pooled samples, as quality control (QCs), n-alkane standards (for retention index calculation), blank solvents (pyridine) were injected at scheduled intervals for instrumental performance, tentative identification, and monitoring of shifts in retention indices.

Raw data (.RAW) were then analysed through the open source software MS DIAL and open source EI spectra library were used for raw peaks extraction, data baseline filtering and calibration, peak alignment, deconvolution analysis, peak identification and integration of the peak height as previously described by Tsugawa et al., (2015). For MS-DIAL data annotations, based on the mass spectral pattern as compared to EI spectral libraries, the following commercial and publicly available libraries were used: NIST Mass Spectral Reference Library (NIST14/2014), MSRI spectral libraries from Golm Metabolome Database (Kopka, 2006), MassBank (Horai et al., 2010) and MoNA (Mass Bank of North America, (http://mona.fiehnlab.ucdavis.edu/).

Once the compounds and features were identified, using the previously mentioned libraries, they were annotated. For metabolite annotation and assignment of the EI-MS spectra, we followed the metabolomics standards initiative (MSI) guidelines (Sumner et al., 2007).

2.6. Statistical analyses

Data were subjected to a one-way analysis of variance (ANOVA) with girdling treatment as the variability factor, and then the means were separated with Fisher's least significant difference (LSD) post-hoc test ($P \leq 0.05$). All the statistical analyses were performed using GraphPad (GraphPad, La Jolla, CA, USA). Data are expressed as mean ± standard deviation. The correlation matrix was carried out using GraphPad software.

Metabolomic data extracted from MSDIAL were statistically analysed using the open source software Metaboanalyst (Chong et al., 2018).

Metabolite concentrations were checked for integrity and missing values were replaced with a small positive value (the half of the minimum positive number detected in the data). Data were successively normalis~~z~~ed by the internal standard ribitol, transformed through "Log normalis~~z~~ation" (to make the metabolite concentration values more comparable among different compounds) and scaled through Pareto-Scaling (values were mean-centered and divided by the square root of standard deviation of each variable). Data were then classified through Principal Component Analysis getting the score plots, to visualis~~z~~e the contrast between different samples, and the loading plots to explain the cluster separation.

3. Results

3.1. Fruit morpho-metric and quality parameters

The fruit average width, height and weight were positively influenced by Girdling 4W treatment showing higher values with respect to both the control and Girdling 2W (~~(~~6~~6~~ and ~7 % of width, ~6 and ~5~~6~~ % of height and ~~24~~ 24 and ~25 % of weight more than control and Girdling 2W, respectively~~, respectively~~; ~~$P \leq 0.01$~~ $P \leq 0.01$; Table 1).

The fruit quality parameter SSC increased in Girdling 2W fruits and even more in those belonging to the 4W treatment respect to control fruit (8 and 14 % respectively; ~~$P \leq 0.001$~~ $P \leq 0.001$; Table 1). In terms of DM in fruit pulp and skin, control and girdled fruits showed only significant differences ~~($P \leq 0.05$)~~($P \leq 0.05$) in fruit pulp, for which higher values (~10 %) were measured in both Girdling 2W and 4W treatments with respect to control fruits (Table 1).

3.2. Colour changes in fruit pulp and skin

Colorimetric CIELab results are summarised in Table 2. Values of L* measured in fruit pulp were negatively influenced by girdling treatment ~~($P \leq 0.001$)~~($P \leq 0.001$), showing lower

values than controls, depending on the duration of treatment (-27 and 47 % in 2W and 4W Girdling, respectively). Conversely, in fruit skin, only the Girdling 4W treatment showed lower values in L* (~47 %) with respect to both control and 2W treatment ~~($P \leq 0.001$)~~($P \leq 0.001$).

Values of a* were higher ~~($P \leq 0.001$)~~ ($P \leq 0.001$) in pulp of fruits belonging to both the girdling treatments (~42 %) when compared to controls, whereas only Girdling 2W fruits showed higher ~~($P \leq 0.001$)~~ ($P \leq 0.001$) values of a* (14 %) in the skin with respect to both control and Girdling 4W fruits.

In fruit pulp, b* parameter was lower ~~($P \leq 0.001$)~~($P \leq 0.001$) in Girdling 2W and even more in 4W treatment (25 and 49 %, respectively) with respect to the control. In fruit skin, control and Girdling 4W showed lower b* values ~~($P \leq 0.001$)~~ ($P \leq 0.001$) than Girdling 2W (27 and 60 %, respectively).

Values of C* detected in fruit pulp decreased in relation to the duration of girdling treatment ~~($P \leq 0.001$)~~($P \leq 0.001$), compared to control fruits (8 and 20 % in 2W and 4W, respectively). In fruit skin, Girdling 2W and 4W showed the highest and the lowest C* values, respectively ~~($P \leq 0.001$).~~ ($P \leq 0.001$).

The h_{ab} values decreased in fruit pulp along with the duration of treatment with respect to the control (28 and 45 % in 2W and 4W, respectively; ~~$P \leq 0.001$~~ $P \leq 0.001$), while in fruit skin only Girdling 4W had lower values then the control and Girdling 2W (~45%) fruits ~~($P \leq 0.001$)~~($P \leq 0.001$).

According to CIE XYZ model, the treatments differed in chromaticity coordinates (x and y) respect to the control both in fruit skin and pulp (Fig. 1A,B). The CIE diagram showed that the control fruit pulp x, y values fall in the orange region whereas those of Girdling 2W and 4W treatments in the reddish-orange one. Fruit skin x, y values of all the three treatments fall in the same red region.

3.3. Pigment analysis in fruit pulp and skin

TA, Chl_{TOT}, and Car contents in fruit pulp and skin are summarised in Table 3. TA content increased markedly ~~($P \leq 0.001$)~~ ($P \leq 0.001$) along with the duration of girdling treatment in both fruit pulp and skin respect to the control (50 and 100 % in pulp of fruit from ~~plants~~ branches subjected to 2W and 4W, respectively; 24 and 64 % in skin of fruit from branches ~~plants~~ subjected to 2W and 4W, respectively). No changes in Chl_{TOT} content were detected in fruit pulp among treatments, whereas in fruit skin, both ~~the~~ girdling treatments showed lower values ~~($P \leq 0.01$)~~ ($P \leq 0.01$) than the control fruits (~22 %). No significant differences were detected in Car content in both fruit pulp and skin among treatments.

Significant positive and negative correlations between TA, quality and colour parameters in fruit pulp and skin were found (Fig. 2). In fruit pulp, TA content was positively correlated with a*, DM and SSC ~~($P \leq 0.001$)~~ ($P < 0.001$) and negatively correlated with L*, b*, C* and h_{ab} ($P < 0.001$). In fruit skin, the TA content was only positively correlated with SSC and negatively correlated with L*, h_{ab} ($P < 0.001$) and b* ($P < 0.01$).

3.4. Total antioxidant activity changes in fruit pulp and skin

~~Total antioxidant activity (~~TAA~~)~~ data are summarised in Fig 3. In fruit pulp, only in Girdling 4W was detected a significant increase in TAA (66 %) respect to the control ~~($P \leq 0.001$)~~($P \leq 0.001$). A similar pattern was showed in fruit skin with higher TAA values (31 %) registered in Girdling 4W respect to the control ~~($P \leq 0.05$)~~($P \leq 0.05$). Moreover, in each treatment, TAA levels were higher in skin respect to pulp (values compared by Student's t-test, $P < 0.05$).

3.5. Metabolic alteration in pulp and skin of fruits under different girdling treatments

To get more insights into metabolome modulation induced by girdling treatment, GC/MS-driven untargeted-metabolomic analysis was performed on both pulp and skin data, allowing to identify and annotate 112 and 81 metabolites (amino acids, organic acids, phenols, sugars, sugar alcohols, amino acid derivatives and miscellaneous) in the pulp and skin (Supplementary Table A.1 and A.2), respectively.

~~Metabolomic data in fruit pulp were analyzed through principal component analysis (PCA).~~ In Fig 4A is reported the PCA score plot, which allowed samples separation basing on their metabolite profiles. Instead, the Fig 4B reported the PCA loading plot that allowed the identification of metabolites that contributes to samples separation reported on the score plot. Groups separation was achieved using the principal components (PCs) PC1 *vs* PC2, which explained a total variance of 57.9 %. In particular, PC1 and PC2 explained the 35.5 % and 22.4 % of the variance, respectively. In addition, the loading plots demonstrated that the PC1 was dominated by GABA, nonacosane, L-alanine and adenosine, whereas the PC2 by L-tryptophan, L-proline and oxalic acid (Fig 4B and Supplementary Table A.3).

As well as the pulp also in the skin the PCA analysis showed a clear separation among all treatments (Fig. 4C), highlighting that the principal components (PCs) PC1 (42 %) *vs* PC2 (25.5 %) explained a total variance of 67.5 % (Fig 4C). The PCA loading plot in Fig 4D shows that the PC1 was mainly dominated by maltitol, benzoic acid, trisaccharide, L-threonine and norvaline whereas the PC2 by fructose, methylsuccinic acid, trehalose, mannitol, H-1ndole-3-acetamide, dehydroascorbic acid, Epicatechin and galactose-6-phosphate and arabitol (Fig. 4D, Supplementary Table A.4).

The univariate analysis one-way ANOVA using the LSD test as post hoc ($P \leq 0.05$) was carried out on every individual compound identified in both skin and pulp. To control for false positive findings, a False Discovery Rate (FDR) was applied on the nominal *p*-values showing that there are 63 and 51 significant compounds (amino acids, organic acids, phenols, sugars,

sugar alcohols, amino acid derivatives and miscellaneous) with *p*-value lower than 0.05 (after the FDR correction) in pulp and skin, respectively (Tab. 4, Supplementary Table A.5 and A.6).

In fruit pulp, Girdling 4W increased 30 polar primary metabolites (5 amino acids, 8 organic acids, 12 sugars and 5 sugar alcohols), decreasing 9 (2 amino acids, 5 organic acids, 1 sugar and 1 sugar alcohol) respect to the control; whereas 32 polar primary metabolites (3 amino acids, 13 organic acids, 10 sugars, 3 sugar alcohols and 3 amino acid derivatives) were increased in Girdling 2W and only one (shikimic acid) was decreased respect to the control. Girdling treatments induced the accumulation of several soluble sugars and sugar alcohols (e.g. sucrose, trehalose, xylitol, inositol), amino acids (e.g. proline, b-alanine), organic acids (citric acid, malic acid, a-ketoglutaric acid). To note that the duration of girdling as well as the fruit stage in which girdling was done, differently influenced the group of accumulated polar primary metabolites (Tab. 4). In fruit pulp, among detected phenols chlorogenic acid, epicatechin were positively affected by Girdling 4W and, whereas pyrogallol only by 2W treatment.

In fruit skin, contrasting effects between girdling treatments were detected. In Girdling 4W, 31 polar primary metabolites (7 amino acids, 10 organic acids, 6 sugars, 6 sugar alcohols and 2 amino acid derivatives) were increased while 4 (3 organic acids and 1 sugar) decreased respect to the control. In Girdling 2W, 11 polar primary metabolites (2 amino acids, 4 organic acids, 1 sugar and 3 sugar alcohol and 1 amino acid derivatives) were increased and 16 (4 amino acids, 4 organic acids, 4 sugars, 2 sugar alcohols and 2 amino acid derivatives) decreased respect to the control. In particular, epicatechin and ferulic acid were positively affected by both girdling treatments respect to the control (Table 4).

4. Discussion

4.1. Girdling influences on quality fruit traits

Girdling is a well-known agronomical practice used in different fruit-crop species such as grape, cherry and, peaches to increase the fruit quality traits. However, there is very little information on the physiological effects exerted by this treatment in plum species (Day and DeJong, 1998; Ilha et al., 1999; Neeraj, 2011). The first goal of this work was the evaluation of the morpho-metric and quality feature (i.e. dimensions, SSC, DM and colour) changes exerted by 2 and 4W-long girdling treatment.

The improvement in fruit size is a common phenomenon induced by girdling already reported for plum or other fruit species belonging to the *Prunus* genus (Day and DeJong, 1998; Neeraj, 2011; Moscatello et al., 2017; Michailidis et al., 2020). Nevertheless, Day and DeJong, (1998) raised for plums the problem of girdling timing; given that a late girdling may not affect the fruit size, the suggestion was to apply the girdling at a very early stage (e.g. after petal-fall). However, our experiments, in which both girdling treatments were applied in late stage of fruit development (4- and 2-weeks before the harvest, namely 'final swell' stage), it was observed that 4W branch girdling had a positive effect on fruit size, whereas the girdling performed in a later stage of fruit development (2-weeks before the harvest) did not affect the fruit size, likely because it was too close to the harvest time to promote such a macroscopic fruit response.

The fruit represents a strong plant sink, and girdling conditions can favour an 'extra' accumulation of soluble solids in fruit above the girdling site by limiting the sink competition for sugars (e.g. other fruits, new shoots or branches), as also confirmed by our experimental analysis and in accordance with previous works (Di Vaio et al., 2001; Goren et al., 2010).

The increase in fruit DM, especially in the pulp is another factor linked to the increased availability of carbohydrates to fruit, the only strong active sink, since no significant increase in wood DM was observed in girdled branches (Supplementary Fig. A.1).

4.2. Fruit colour changes due to girdling

Fruit colour is a well-appreciated trait by consumers, which is dependent on both the maturity stage as well as changes in pigment pattern (e.g. chlorophyll degradation and anthocyanin accumulation) due to environmental factors, which might be not directly related to the ripening development. In particular, ripe plums of *Prunus cerasifera* var. *pissardii* reached a very attractive dark-red colouration on skin and in pulp at full maturity. Moreover, the fruit skin appearance can strongly influence the consumer choice, and especially the skin colour is one of the most important traits to determine the fruit aesthetic value (Kayesh et al., 2013).

In our experiment, girdling treatment influenced the pigment composition in both fruit pulp and skin, mainly promoting the biosynthesis of anthocyanins that were increased linearly with the girdling duration in both fruit pulp and skin. In particular, the red colouration increased in the pulp ~~(lower values of h_{ab} respect to control)~~. In plum fruit, ~~, as also visually confirmed by chromatic graph (Fig. 1). In the fruit skin, only the Girdling 4W resulted in an appreciable darker red colouration (compared to the control), showing similar trend of chromatic parameters to those measured in the pulp.~~ t~~T~~he increase in reddish colouration ~~increment in plum~~ can be ascribed to the increased biosynthesis of red/purple pigments (anthocyanins) and/or the degradation of other coloured molecules such as chlorophylls and carotenoids (Olivares et al., 2017). Indeed, under 2W girdling treatment, the anthocyanin increase in the fruit skin was too low to be detected. The anthocyanin increase induced by girdling was reported in some fruits , e.g. in grapes (Basile et al., 2018) red kiwifruit (Nardozza et al., 2018) and cherries (Michailidis et al., 2020). The increase in anthocyanins was positively correlated with SSC and DM, suggesting a strictly link between anthocyanin and soluble sugar content as discussed in the next paragraphs.

Besides anthocyanins, chlorophylls and carotenoids are other pivotal pigments in contributing to fruit colouration. Girdling treatment also significantly induced the decrease in chlorophyll concentration in fruit skin respect to the control fruits. The higher chlorophyll degradation could be due to an advanced fruit ripe stage given by girdling treatment, that promotes the chlorophyll breakdown (Kato and Shimizu, 1985; Hörtensteiner and Kräutler, 2011).

4.3. Metabolic alteration in pulp

In the fruit ripening process, complex coordination changes among metabolites (e.g. sugars, organic acids and amino acids) take place influencing flavour and organoleptic fruit characteristics (Batista-Silva et al., 2018).

In fruit pulp, several sugars (e.g. sucrose, trehalose and galactose-6-phosphate) and sugar alcohols (e.g. inositol and xylitol) were mostly accumulated after girdling treatments respect to control and this effect was more marked (prominent) with a later treatment (Girdling 4W ~~vs~~ *vs* Girdling 2W). The accumulation of sugars and sugar alcohols have long been found in plant tissues above the girdling area (Goren et al., 2010; Michailidis et al., 2020). ~~According to, in both 4W and 2W fruits,~~ M~~m~~any organic acids, especially some TCA cycle intermediates such as α-ketoglutaric, citric, isocitric, fumaric and malic acid were accumulated in both 4W and 2W fruits. These results suggest an upregulation of the respiratory metabolism promoted by girdling; the oxidation of carbohydrates (that are accumulated at higher quantity in girdled treatments respect to the control) via glycolysis provides carbon skeleton for the TCA cycle contributing to the generation of their intermediates (organic acids) (Batista-Silva et al., 2018). Moreover, in leaves Figueroa et al. (2016) proposed a sugar sensing mechanism that stimulates the anaplerotic synthesis of organic acids and it is possible that the girdling treatment influences this network. Organic acids, in turn, can be used for the biosynthesis of amino acids (Miflin

and Lea, 1977), for which an increase of β-Alanine, GABA, L-Leucine, L-Proline and L-Serine content was detected mainly in Girdling 4Wwith respect to the control. The accumulation of amino acids in the pulp ~~accumulation of~~to girdled fruits can be potentially related to different coexisting physiological phenomenon: i) their reduced use in cellular biosynthetic processes; and ii) a strong phloem sap (which also contains amino acids; Famiani et al., 2012) translocation to the fruit (the only active sink which was studied in girdling conditions in the present experiments). Despite the metabolic picture described above, in not-girdled conditions ~~in normal condition~~ throughout the fruit ripening, in the majority of fleshy fruits, sugar content normally increases (due to fruit starch degradation and the import of sugars from source tissues), whereas the level of organic acids and amino acids tends to decrease (Nardozza et al., 2013; Batista-Silva et al., 2018; Jiang et al., 2019). The results suggested that girdling treatment increased several organic acids and amino acids biosynthesis in fruit pulp, in accordance with other works in which an increase in these compounds especially those related to TCA cycle was detected (Basile et al., 2018; Michailidis et al., 2020). However, in these works emerge that fruit variety as well as the fruit developmental stage (at which girdling is performed) can strongly influence the primary metabolism responses to the girdling treatment.

Another physiological consequence due to girdling, related to the sugars increase , is the increase in anthocyanin biosynthesis in fruit tissue (Basile et al., 2018; Nardozza et al., 2018; Michailidis et al., 2020). Anthocyanins constitute one of the end-branch of phenol pathway, formed by the glycosylation (through glucosyltransferase UFGT enzyme action) of anthocyanidin with one or more sugars (Silva et al., 2016). At the molecular level, anthocyanins can be induced by sugar signalling network (Das et al., 2012; Solfanelli et al., 2006). Therefore, the sugars accumulation in fruit pulp under girdling conditions likely induced the anthocyanin biosynthesis making the pulp more reddish than that of control. Anthocyanins share the same metabolic intermediates with another group of phenols, epicatechins (Mouradov and

Spangenberg, 2014). Epicatechins are produced by the reduction of anthocyanidin through anthocyanidin reductase (ANR) enzyme. A molecular cross-talk between epicatechins and anthocyanins production was reported for some plant species, since these two molecules need the same substrate (anthocyanidin). Ectopic expression of ANR resulted in an accumulation of epicatechins with a consequent reduction of anthocyanins in transgenic tobacco plants (Xie, 2003), whereas downregulation of ANR induced an accumulation of anthocyanins in plant tissue (Fischer et al., 2014; Mouradov and Spangenberg, 2014). This could in part explain the increase in anthocyanins in pulp with a parallel reduction in epicatechins as observed in Girdling 2W.

4.4. Metabolic alteration in skin

The fruit skin is the primary fruit defence line, since it represents the interface between fruit and its environment, constituting a physio-chemical barrier to biotic and abiotic stressors such as pathogens, drought and UV light. For these reasons, fruit skin is very rich in phytochemical compounds more than pulp including phenols, ascorbic acid, glutathione and others antioxidant enzymes (Li et al., 2008; Cosmulescu et al., 2015). At the same time, as for consumers, fruit skin features are essential for animal attraction, which is essential for species propagation.

Rarely the metabolic changes in fruit skin after girdling treatment have been studied (Yang, 2009), since usually skin represents a fruit aesthetic trait and only colour changes were measured (Agusti et al., 1998; Simmons et al., 1998; Ren et al., 2013). However, in fruits with edible skin, the detection of changes in metabolite content can be useful to understand the physiological responses to girdling, and also to improve fruit nutritional qualities. As a matter of ~~fact~~fact~~,s,~~ the metabolomic traits of fruit skin in both girdling treatments were perturbed respect to the control, even if these effects were more evident in the Girdling 4W respect to the

2W one. In particular, Girdling 4W induced major change in sugar contents with consequent accumulation of sugars (e.g. glucose 6-phosphate and fructose 6-phosphate) and sugar alcohols (sorbitol 6-phosphate, xylose and xylitol). Furthermore, many organic acids related to TCA cycle (a-ketoglutaric acid, citric acid and succinic acid) and amino acids related or not to TCA cycle (e.g. asparagine, threonine, L-Isoleucine, b-Alanine and L-Phenylalanine) were increased in Girdling 4W, suggesting a similar trend and explanation proposed for pulp tissue. Specifically, the increase in L-Phenylalanine, a substrate of phenylalanine ammonia-lyase (a key enzyme of phenolic metabolism) (MacDonald and D'Cunha, 2007), with a parallel increase in polyphenols (chlorogenic acid, epicatechin, ferulic acid and anthocyanins) in Girdling 4W can likely suggest a stimulation of secondary metabolism due to the girdling treatment. However, results showed an increase in anthocyanin content in both treatments. Though in each treatment, skin TA were ~4-fold higher than values found in pulp, their increase was lower in percentage when compared to that detected in the pulp, probably because also epicatechins increased in both treatments constituting a competitor for their biosynthesis.

Girdling also had a positive effect on ascorbic acid content in skin respect to the control increasing also its oxidised form the dehydroascorbic acid. Ascorbic acid is one of the most abundant antioxidants (Li et al., 2008), and ~~his~~ its increase in skin tissues can provide a further fruit resistance to oxidative stresses ~~given by girdling~~. However, remains unclear whether its increase is due to increased soluble sugar availability stimulated ~~his~~ its biosynthesis or to an increased import from foliage or other fruit tissues (Li et al., 2008; Yang et al., 2013).

The focus on primary metabolomic changes induced by girdling gave us a partial picture of skin metabolism. Further study on skin secondary metabolism profile (that probably have major importance in the skin than pulp) needs to be addressed. However, as shown by the present dataset, skin physiological responses and potential metabolic changes to girdling seem to be closely related to the girdling time.

4.5. Girdling influences on total antioxidant activity in pulp and skin

There are several biological reasons that support the health-promoting effects given by fruits consumption. Fruits are rich in fibres, carbohydrates, vitamins and bioactive compounds characterised by high antioxidant properties (Del Río-Celestino and Font, 2020). Girdling applied at different time before harvest of the fruit differentially increased several bioactive molecules (e.g. anthocyanins, chlorogenic acid, epicatechin, ferulic acid and ascorbic acid) as described in the previous paragraph in both fruit pulp and skin. However, it worth to be highlighted that only Girdling 4W showed a relative increase in TAA respect to control in both pulp and skin. This shows that even though the concentration of several biologically-active molecules were increased in both treatments, a longer girdling treatment (in which these compounds were more accumulated) is required to increase the TAA in the fruit. In accordance with a very recent research by Michailidis et al (2020), the dataset presented herein confirms that girdling is a valid treatment to enhance the biosynthesis of bioactive molecules, which could be proficiently exploited for the production of 'Nutrafruits'.

5. Conclusion

The hypothesis that girdling influences fruit characteristics, primary metabolism, anthocyanin level and antioxidant activity in *Prunus cerasifera* var. *pissardii* fruit was confirmed. In our experiment, the girdling treatment influenced the normal fruit metabolic dynamics in both skin and pulp by inducing accumulation of sugars, sugar alcohols, organic acids, amino acids, anthocyanins and other phenols. Girdling 4W was the treatment that led to more positive changes in fruits (under qualitative and nutritional aspects) than Girdling 2W. This approach could be used with other commercial fruit varieties and species in order to help

growers to ensure a production of red-fleshed fruits with a homogeneous red coloration and higher antioxidant properties. Moreover, results of the present manuscript reinforce the idea that new researches should be conducted to understand possible further influences given by different girdling dates, in order to understand when to perform the girdling to obtain fruits with high-quality characteristics, since also a late girdling induced strong responses in *P. cerasifera* var. *pissardii* fruits.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Founding

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Appendix A. Supplementary data

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Legends of figures

Figure 1. Chromaticity coordinates of control (open circles), 4-weeks (before the harvest) girdling (open squares) and 2-weeks (before the ~~girdling~~harvest) girdling (open triangles) in pulp (A) and skin (B) of plum fruits (*Prunus cerasifera* var. *pissardii*).

Figure 2. Pearson's correlation coefficient between total anthocyanin concentration (TA), CIELab ~~parameters~~lightness (L*), redness and greenness (a* and –a*), yellowness and blueness (b* and –b*), chroma (C*) hue angle (~~(L*, a*, b* C* and~~ h_{ab}), dry matter (DM) and soluble solid content (SSC) in pulp and skin of treated and control plum fruits (*Prunus cerasifera* var. *pissardii*). *: $P < 0.05$. **: $P < 0.01$; ***: $P < 0.001$.

Figure 3. Total antioxidant activity (TAA) determined in control (open bars), 4-weeks (before the harvest) girdling (closed bars) and 2-weeks (before the harvest) girdling (semi-closed bars) pulp (A) and skin (B) of plum fruits (*Prunus cerasifera* var. *pissardii*). The mean values (±SD; $n = 4$) were subjected to one-way ANOVA with treatment as the source of variations. Means flanked by the same letter are not statistically different for $P = 0.05$ after Fisher's least significant difference post-hoc test.

Figure 4. Principal component analysis (PCA) score and loading plot determined in pulp (A,B) and skin (C,D) of plum fruits (*Prunus cerasifera* var. *pissardii*) by using the metabolome profile of control, 4-weeks (before the harvest) girdling and 2-weeks (before the harvest) girdling treatments.

Tables

Table 1.

		Fruit		
Parameters	Units	Control	Girdling 4W	Girdling 2W
Width	mm	21.47 ± 1.06[b]	22.69 ± 0.75[a]	21.13 ± 0.76[b]
Height	mm	20.58 ± 1.12[b]	21.74 ± 1.08[a]	20.75 ± 0.95[b]
Weight	g	5.32 ± 0.66[b]	6.61 ± 1.05[a]	5.29 ± 0.34[b]
SSC	°Brix	11.8 ± 0.74[c]	13.5 ± 1.2[a]	12.7 ± 1.2[b]
DM (Pulp)	%	24.09 ± 2.40[b]	26.55 ± 3.08[a]	26.15 ± 2.64[a]
DM (Skin)	%	43.85 ± 4.95	45.99 ± 9.69	45.66 ± 6.82

Morphological and quality parameters in Control, 4-weeks (before the harvest) girdling and 2-weeks (before the harvest) girdling of plum fruits (*Prunus cerasifera* var. *pissardii*). The mean values (±SD; n = 20) were subjected to one-way ANOVA with treatment as the source of variations. Means flanked by the same letter are not statistically different for P = 0.05 after Fisher's least significant difference post-hoc test.

Table 2.

	Pulp			Skin		
Parameters	Control	Girdling 4W	Girdling 2W	Control	Girdling 4W	Girdling 2W
L*	27.02 ± 6.92[a]	14.32 ± 3.94[c]	19.53 ± 6.25[b]	10.75 ± 3.29[a]	5.46 ± 2.24[b]	11.25 ± 3.47[a]
a*	23.04 ± 8.52[b]	33.12 ± 6.87[a]	32.49 ± 4.15[a]	32.29 ± 5.90[b]	30.30 ± 4.19[b]	36.97 ± 2.30[a]
b*	44.07 ± 9.54[a]	22.58 ± 8.09[c]	32.80 ± 8.00[b]	12.58 ± 6.39[b]	6.76 ± 3.72[c]	17.22 ± 7.44[a]
C*	50.89 ± 6.43[a]	40.79 ± 7.29[c]	46.64 ± 5.95[b]	34.93 ± 7.50[b]	31.19 ± 4.72[c]	41.21 ± 4.79[a]
h_{ab}	61.76 ± 13.35[a]	33.98 ± 11.69[c]	44.59 ± 8.65[b]	20.33 ± 6.88[a]	12.03 ± 5.58[b]	24.11 ± 8.43[a]

CIELAB parameters: lightness (L*), redness (a*), yellowness (b*), chroma (C*) and hue angle (h_{ab}) of pulp and skin in Control, ~~42~~-weeks (before the harvest) girdling and 2-weeks (before the harvest) girdling of plum fruits (*Prunus cerasifera* var. *pissardii*). The mean values (±SD; n = 20) were subjected to one-way ANOVA with treatment as the source of variations. Means flanked by the same letter are not statistically different for P = 0.05 after Fisher's least significant difference post-hoc test.

Table 3.

Parameters	Units (FW)	Pulp			Skin		
		Control	Girdling 4W	Girdling 2W	Control	Girdling 4W	Girdling 2W
TA	mg g^{-1}	0.08 ± 0.02^{c}	0.16 ± 0.03^{a}	0.12 ± 0.03^{b}	0.37 ± 0.13^{c}	0.61 ± 0.09^{a}	0.46 ± 0.09^{b}
Chl_{TOT}	mg 100 g^{-1}	0.72 ± 0.12	0.73 ± 0.11	0.68 ± 0.07	6.70 ± 0.44^{a}	4.91 ± 0.87^{b}	5.56 ± 0.38^{b}
Car	mg 100 g^{-1}	0.83 ± 0.13	1.02 ± 0.07	0.93 ± 0.13	3.79 ± 0.56	4.19 ± 0.53	3.90 ± 0.29

Total anthocyanins (TA), total chlorophylls (Chl_{TOT}) and total carotenoids (Car) of pulp and skin in Control, 4-weeks (before the harvest) girdling and 2-weeks (before the harvest) girdling of plum fruits (*Prunus cerasifera* var. *pissardii*). The mean values (±SD; $n = 20$) were subjected to one-way ANOVA with treatment as the source of variations. Means flanked by the same letter are not statistically different for $P = 0.05$ after Fisher's least significant difference post-hoc test.

Table 4.

	Pulp			**Skin**			
Compounds	**Control**	**Girdling 4W**	**Girdling 2W**	**Control**	**Girdling 4W**	**Girdling 2W**	**Classes**
Alanine	0.346^{a}	0.017^{b}	0.046^{a}	-	-	-	***Amino acids***
Asparagine	-	-	-	0.264^{b}	0.350^{a}	0.345^{a}	
ß-Alanine	0.216^{c}	0.275^{a}	0.242^{b}	0.156^{b}	0.211^{a}	0.138^{c}	
b-Cyano-L-Alanine	0.012^{a}	0.008^{b}	0.014^{a}	-	-	-	
GABA	0.852^{b}	2.174^{a}	0.874^{b}	0.881^{b}	2.017^{a}	0.966^{b}	
L-Aspartic acid	-	-	-	0.240^{b}	0.227^{b}	0.309^{a}	
L-Glutamic acid	-	-	-	0.337^{a}	0.304^{a}	0.250^{b}	
L-Isoleucine	-	-	-	0.002^{b}	0.004^{a}	0.002^{b}	
L-Leucine	0.003^{b}	0.007^{a}	0.002^{b}	-	-	-	
L-Lysine	0.003^{b}	0.004^{ab}	0.005^{a}	-	-	-	
L-Norvaline	0.113^{ab}	0.182^{a}	0.060^{b}	0.027^{b}	0.060^{a}	0.024^{b}	
L-Phenylalanine	-	-	-	0.044^{b}	0.055^{a}	0.031^{c}	
L-Proline	0.005^{b}	0.080^{a}	0.052^{a}	-	-	-	
L-Serine	0.622^{b}	0.810^{a}	0.652^{b}	0.073^{a}	0.082^{a}	0.043^{b}	
L-Threonine	-	-	-	0.046^{b}	0.121^{a}	0.043^{b}	
α-Ketoglutaric acid	0.632^{b}	0.682^{a}	0.699^{a}	0.791^{b}	0.938^{a}	0.775^{b}	***Organic acids***
Aconitic acid	0.029^{b}	0.028^{b}	0.042^{a}	-	-	-	
Citramalic acid	0.010^{b}	0.008^{c}	0.012^{a}	0.015^{b}	0.023^{a}	0.016^{b}	

Citric acid	0.002^{b}	0.002^{a}	0.002^{a}	1.110^{b}	1.612^{a}	0.940^{b}	
Dehydroascorbic acid	-	-	-	0.034^{c}	0.049^{b}	0.074^{a}	
DL-Isocitric acid	0.019^{b}	0.026^{a}	0.020^{b}	-	-	-	
Fumaric acid	0.897^{b}	0.760^{c}	1.25^{a}	1.271^{a}	1.178^{ab}	1.052^{b}	
Gluconic acid	0.058^{b}	0.060^{b}	0.075^{a}	0.116^{b}	0.095^{c}	0.134^{a}	
Glutaric acid	0.025^{c}	0.037^{a}	0.031^{b}	0.031^{b}	0.040^{a}	0.030^{b}	
Glycolic acid	-	-	-	0.025^{b}	0.031^{a}	0.015^{c}	
Isocitric acid minor	0.084^{c}	0.100^{b}	0.109^{a}	-	-	-	
L-ascorbic acid	-	-	-	0.027^{b}	0.040^{a}	0.045^{a}	
L-Tartrate	0.002^{a}	0.001^{b}	0.002^{a}	0.001^{b}	0.001^{a}	0.001^{b}	
Maleic acid	2.78^{b}	2.63^{b}	3.44^{a}	-	-	-	
Malic acid	0.261^{b}	0.290^{a}	0.305^{a}	-	-	-	
Malonic acid	0.005^{a}	0.004^{b}	0.006^{a}	-	-	-	
Methylsuccinic acid	-	-	-	0.030^{a}	0.016^{b}	0.013^{b}	
Phosphoric acid	0.878^{b}	0.990^{a}	1.019^{a}	-	-	-	
Quinic acid	2.650^{c}	2.887^{b}	3.106^{a}	-	-	-	
Shikimic acid	1.438^{a}	0.879^{c}	1.341^{b}	2.087^{a}	1.692^{b}	1.722^{b}	
Succinic acid	-	-	-	1.703^{b}	2.213^{a}	1.594^{b}	
Threonic acid	0.559^{b}	0.591^{b}	0.655^{a}	0.111^{b}	0.146^{a}	0.139^{a}	
3,4-Dihydroxybenzoate	-	-	-	0.055^{c}	0.071^{a}	0.063^{b}	***Phenols***
Benzoic acid	-	-	-	0.001^{b}	0.005^{a}	0.001^{b}	
Cafferic acid	0.007^{a}	0.005^{b}	0.011^{a}	0.041^{b}	0.034^{b}	0.055^{a}	
Chlorogenic acid	0.002^{b}	0.005^{a}	0.002^{b}	0.021^{a}	0.019^{a}	0.014^{b}	
Epicatechin	0.066^{b}	0.077^{a}	0.029^{c}	0.049^{c}	0.154^{a}	0.109^{b}	
Ferulic acid	-	-	-	0.005^{b}	0.006^{a}	0.006^{a}	
Pyrogallol	0.006^{b}	0.005^{b}	0.008^{a}	-	-	-	
α-Lactose	0.565^{b}	0.579^{b}	0.664^{a}	-	-	-	***Sugars***
ß-Gentibiose	0.152^{c}	0.216^{a}	0.180^{b}	-	-	-	
ß-Lactose	0.048^{b}	0.064^{a}	0.062^{a}	-	-	-	
D-Lyxose	0.946^{b}	1.123^{a}	1.018^{ab}	-	-	-	
D-Ribose	-	-	-	0.013^{a}	0.017^{a}	0.006^{b}	
D-Xylose	-	-	-	1.013^{b}	1.132^{a}	0.973^{b}	
Fructose-6-phosphate	-	-	-	0.070^{b}	0.118^{a}	0.081^{b}	
Galactose-6-phosphate	0.158^{c}	0.184^{b}	0.206^{a}	0.000^{c}	0.000^{b}	0.000^{a}	
Gentibiose	0.070^{b}	0.130^{a}	0.071^{b}	-	-	-	
Glucoheptulose	0.004^{c}	0.006^{a}	0.004^{b}	-	-	-	
Glucose-1-phosphate	0.489^{a}	0.376^{b}	0.510^{a}	-	-	-	
Glucose-6-phosphate	-	-	-	0.113^{b}	0.199^{a}	0.122^{b}	
Maltose	0.112^{b}	0.107^{b}	0.128^{a}	-	-	-	
Maltotriose	0.001^{b}	0.002^{a}	0.001^{a}	-	-	-	
Melbiose	0.133^{b}	0.308^{a}	0.145^{b}	0.171^{b}	0.222^{a}	0.121^{c}	
Ribose	0.274^{b}	0.285^{b}	0.338^{a}	-	-	-	
Sorbose	1.269^{b}	1.536^{a}	1.348^{b}	-	-	-	
Sucrose	0.019^{c}	0.029^{a}	0.026^{b}	-	-	-	
Trehalose	0.002^{b}	0.004^{a}	0.003^{a}	0.121^{a}	0.066^{b}	0.054^{c}	
Trisaccharide	0.000^{b}	0.000^{a}	0.000^{b}	0.006^{b}	0.013^{a}	0.003^{c}	
Arabitol	-	-	-	0.003^{b}	0.006^{a}	0.006^{a}	***Sugar alcohols***
Galactinol	0.000^{b}	0.000^{a}	0.000^{b}	0.038^{b}	0.053^{a}	0.032^{c}	
Galactitol	0.365^{a}	0.214^{b}	0.346^{a}	-	-	-	
Inositol	1.743^{c}	1.898^{b}	2.051^{a}	-	-	-	

Lactitol	0.045^{b}	0.079^{a}	0.047^{b}	0.213^{b}	0.245^{a}	0.160^{c}	
Maltitol	0.106^{b}	0.220^{a}	0.110^{b}	0.073^{b}	0.173^{a}	0.077^{b}	
Mannitol	0.502^{ab}	0.473^{b}	0.529^{a}	-	-	-	
Sorbitol	0.750^{b}	0.777^{b}	0.892^{a}	-	-	-	
Sorbitol-6-phosphate	-	-	-	0.005^{c}	0.012^{a}	0.007^{b}	
Xylitol	0.635^{c}	0.748^{a}	0.672^{b}	0.573^{b}	0.699^{a}	0.736^{a}	
3-Amino isobutyric acid	0.035^{b}	0.037^{ab}	0.039^{a}	0.032^{b}	0.038^{a}	0.024^{c}	***Amino acid derivatives***
Pyroglutamic acid	0.449^{b}	0.445^{b}	0.732^{a}	0.834^{b}	0.914^{b}	1.077^{a}	
Putrescine	0.147^{b}	0.152^{b}	0.184^{a}	0.129^{b}	0.163^{a}	0.112^{c}	
2-Aminoethanol	0.121^{b}	0.130^{b}	0.144^{b}	0.087^{b}	0.109^{a}	0.053^{c}	***Miscellaneous***
3-Aminopropionitrile fumarate	-	-	-	0.005^{a}	0.003^{b}	0.004^{b}	
4-Hydroxybenzoic acid	0.003^{b}	0.007^{a}	0.003^{b}	-	-	-	
4-Hydroxybutyric acid	-	-	-	0.001^{b}	0.002^{a}	0.001^{c}	
6-Phosphogluconic acid	0.007^{c}	0.014^{a}	0.010^{b}	-	-	-	
Adenosine	0.002^{a}	0.000^{b}	0.003^{a}	-	-	-	
ß-mannosylglycerate	0.002^{a}	0.002^{a}	0.002^{b}	-	-	-	
Hydroxylamine	0.572^{b}	0.628^{a}	0.570^{a}	-	-	-	
Tryptamine	-	-	-	0.227^{b}	0.279^{a}	0.216^{b}	

Metabolomics profile of pulp and skin in Control, 4-weeks girdling and 2-weeks girdling of plum fruits (*Prunus cerasifera* var. *pissardii*). The mean values (±SD; $n = 4$) were subjected to one-way ANOVA with treatment as the source of variations. Means flanked by the same letter are not statistically different for $P = 0.05$ after Fisher's least significant difference post-hoc test.