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Research Article

Effect of Cluster Tip Removal of Inflorescences on Phenolics and Antioxidant Activity of Grape Berries and Wines

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Abstract: Insufficient sunlight during veraison to the maturing stage of wine grapes is one of the main factors that inhibits the production of phenolic compounds. In this study, a novel thinning technique called cluster tip removal (CTR) was applied to the inflorescence of *Vitis vinifera* L. Marselan grapes, and the berry composition, phenolic profiles and antioxidant activities of berry skins and wines were determined. The results showed that the CTR of inflorescence resulted in reduced cluster compactness, lower titratable acidity, and higher Brix levels in berries. CTR also increased the concentration of total phenolics, anthocyanins, tannins, and flavonoids in the distal end skins of berries and enhanced the concentrations of 12 phenolic compounds. It also increased the concentrations of total flavanols and total anthocyanins concentrations in wines. The berries and wines in the CTR group were also had higher antioxidant capacities compared with those in the control

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group. Finally, the expression of phenolic-related genes was upregulated in the skin of berries from the CTR group as compared with that from the unpruned group.

Key words: anthocyanin, antioxidant activity, phenolic acid, pruning

Introduction

Phenolic compounds, including anthocyanins, flavan-3-ols, flavonols, phenolic acids and stilbenes, are involved in the growth and development of a wide variety of plants and are intimately involved in their metabolism. For example, phenolic acids can participate in nitrogen metabolism in *Populus* × *euramericana* Neva by acting as an allelochemical inhibitor of its photosynthesis (Xie et al. 2018). Anthocyanins, another class of phenolic compounds, are involved in many aspects of plant growth, including tolerance to ultraviolent (UV)-B damage and drought stress and the elimination of free radicals (An et al. 2019, Wang et al. 2019). Phenolic compounds are abundant in grapes; their concentrations are used to determine the quality and nutritional values of grapes, since they can play roles in preventing oxidative damage and providing health benefits to human (Lingua et al. 2016a). In addition, they can contribute to the mouthfeel and color of red wines (Isabelle and Noble, 2005, Tian et al. 2011). The amounts of phenolic compounds also contribute to the antioxidant capacity and nutritional value of grapes and wine products (Tian et al. 2011, Lingua et al. 2016b).

The degree of variation of phenolic compounds in wine grapes depends substantially on the variety of the grapes and is largely influenced by climates and cultural practices (Rodríguez Montealegre et al. 2006). In the past few decades, many researchers have studied the influence of grape growing environment on phenolic compounds in grape. Pathways in which carbon flows in flavonoid metabolism to a specific branch of grape berries can be influenced by “terroir”, which can include the characteristics of soils and climates, such as sunlight, temperature, rainfall and topography; and among which, the most important influencer is sunlight (Li et al. 2011). One study indicated that sunlight exposure influences the production of phenolic compounds in

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grapes (Spayd and Tarara, 2002). Additionally, exposure to ultraviolet light has also been shown to improve the accumulation of phenolic compounds in grapes (Pan et al. 2009).

Thus far, there has been a rapid expansion of grape and wine production in China, and the top three wineries (among many other wineries) are located in the Jiaodong Peninsula region, one of the most famous regions that has wineries (Changyu, COFCO Great Wall, and Weilong). Wine production, income and profits of the large-scale wineries in Yantai, a city on the Jiaodong Peninsula, accounts for 32.57%, 53.35% and 64.03% of the total domestic wine production, income and profits, respectively. The north of China has a continental monsoon climate, which is rainy and hot year-round. This region has insufficient sunlight, which is the main factor that leads to the low quality of wine grapes (Hua et al. 2009). The contents of anthocyanin, flavonols and phenolic acids in Cabernet Sauvignon grown in this region are lower than those grown in regions in western China, such as those in Xinjiang, Gansu and Ningxia (Li et al. 2011).

To improve the production of secondary metabolites in grapes, vine growers usually remove the leaves around the clusters to improve sunlight exposure of grapes. However, berry clusters of most wine grape cultivars of *Vitis vinifera* are tight, especially for those grown in the Jiaodong Peninsula region, and the berry sizes are large owing to heavy rainfall. In addition, the distal end skins of berries and skins of berries inside the clusters usually have poor colors, which is one of the indicators that the fruit is of poor quality. Reducing cluster compactness by techniques such as berry thinning is an effective way to improve sunlight exposure (Han et al. 2019), but the operation is laborious and extremely time-consuming. The clusters also usually remain cylindrical, and the skins at the bottom of berries still cannot receive sufficient sunlight. Thus, in this study, we attempted to improve the sunlight exposure of the inner berries using cluster tip removal (CTR) of inflorescence technique and evaluated its effects on the phenolic profiles and antioxidant properties of the berries. This work provides a useful cultivation practices that can be applied to optimize the quality of wine and grapes grown in the Jiaodong Peninsula region or other vineyards that have insufficient sunlight.

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Materials and Methods

Plant materials and CTR of inflorescences. Seven-year-old own-rooted 'Marselan' (*Vitis vinifera* L.) vines were used. The study was conducted from 2017 to 2018 during two consecutive seasons in a commercial vineyard (the Great Wall Wine Co., 37°8′N, 120°75′E) in Penglai (Yantai, Shandong, China), which is a seaside county with sandy loam soil (annual average rainfall is 606.2 mm, active accumulated temperature from July to September in the most recent 10 years are 2112 ℃, the cumulative hours of sunshine from July to September during the most recent 10 years are 600-700 h). The daily average temperature, sunlight hours and rainfall during 2017 to 2018 can be found in Table S1. Vines with a vertical trellis system were spaced at 2.5 m (row spacing) × 1 m (vine spacing) in a north–south row orientation.

On June 3 in 2017 and June 7 in 2018, the CTR (cluster tip removal) technique was performed on grapevines at the E-L 17 stage (12 leaves separated; inflorescence well developed, single flowers separated). Specifically, one hand was used to hold up the inflorescence, while the other was used to cut off the upper branches along the rachis with a pair of scissors. Then, depending on the variety's tightness, one or two large lateral branches were cut off at intervals. Finally, approximately one-third of the small flower spikes were cut off, and the remaining clusters were flat. Each inflorescence of a vine was pruned (Figure 1E). In 2020, this CTR technique obtained a Chinese national invention patent (CN 108112420 B).

The experiment followed a randomized block design with three replications. Three blocks with similar texture and fertility in the vineyard were selected, and each replicate consisted of one of 120 vines. In order to reduce marginal effect, guard rows were set between every treatment. The CTR and control samples were harvested at the same time when the Brix of control berries were above 23%. However, in 2017, the weather conditions were bad and had caused the rainfall during the berry development period (July-September) to be 123% higher than that in 2018 (Table S1). Diseases such as downy mildew (*Plasmopara viticola*) and white-rot fungi (*Coniothyrium diplodiella*) caused by the rainfall were also more serious. Thus, in 2017, we harvested the berries in advance of the typical harvest time to reduce losses, which may have caused the insufficient Brix in

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the control berries. In each treatment, a total of 20 clusters of each replicate were collected to determine the berry composition.

Basic maturity parameters of the berries from five clusters of each replicate were determined. The skins of berries from eight clusters of each replicate were divided to two parts: the pedicel end and the distal end (Figure S1). After being divided, the berry skins were immediately frozen in liquid nitrogen and stored at −80 °C before being subjected to phenolic compounds extraction, antioxidant capacity determination and RT-qPCR analysis. The whole skins of berries from seven clusters were peeled and immediately frozen in liquid nitrogen, stored at −80 °C and used to determine the anthocyanin compounds.

Determination of basic maturity parameters. The number of grape berries in each cluster was counted, and the cluster length was measured using a vernier caliper. Cluster weight and berry weight were determined using an electronic scale (Mettler Toledo Instruments Co. Ltd, Shanghai, China). Berry number/cluster length and cluster weight/cluster length ratios were then calculated. The Brix was measured using a hand refractometer (Atago Co. Ltd., Tokyo, Japan). The pH value was determined using a Mettler Toledo FE20 Desktop pH Meter (Mettler Toledo Instruments Co. Ltd). The titratable acidity (TA) content was determined as described in our previous study (Xu et al. 2017). The yield was determined during the harvest. All treatments were carried out in triplicate (n = 3), and the data were averaged.

Extractions of phenolic compounds. Total phenolics, anthocyanins, flavonoids, flavanols, condensed tannins and phenolic compounds were extracted as previously published method (Xu et al. 2017). One gram of grape skin powder or 1 mL of wine was mixed with 8 mL of methanol containing 0.1% acetic acid, and the mixture was then incubated in a water bath at 25 °C for 1 h. After that, the mixture was centrifuged at 8000 rpm at 4 °C for 15 min. The residues were re-extracted three times, and the supernatants were collected and combined before being filtered through a filter paper. The filtrate was evaporated to dryness at 30 °C in a rotary evaporator and then re-dissolved in 5 mL of methanol (HPLC grade). One part of the extract was used for the determination

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122 of total anthocyanins, phenols and flavonoids, while the other part was purified through a C_{18} solid phase
123 extraction (SPE) cartridge (ProElut, DIKMA, China). In the purification process, the cartridges were first rinsed
124 with methanol (HPLC grade). After that, the samples were passed through the cartridges using a vacuum pump
125 operated at a flow rate lower than 0.7 mL/s and were then collected in sample bottles attached to the cartridges.
126 The contents of phenolic compounds in the purified extract were then determined.

127 **Photometric determination of total anthocyanins, phenols, flavonoids, flavanols and condensed**
128 **tannins.** The total anthocyanin concentration was calculated using the equation: OD = A530 – (0.25×A657), in
129 conjunction with a calibration curve that was prepared using standard malvidin-3-monoglucoside
130 (Extrasynthese, Lyon, France) (Li et al. 2013). Total phenols concentration was measured using the Folin-
131 Ciocalteu method (Zhang 2005) and a calibration curve prepared using gallic acid. The total concentration of
132 flavonoids was determined using a colorimetric method described previously (Singleton and Rossi Jr 1965), and
133 a calibration curve was prepared using rutin. The data were expressed as micrograms of rutin equivalent. The
134 measurement of condensed tannin concentration was carried out as previously described (Broadhurst and Jones
135 1978). The total flavanol concentration was calculated using a calibration curve constructed using (+)-catechin
136 as the standard. The content of tannin was measured against a catechin standard curve and expressed as
137 milligrams of catechin equivalent per gram of fresh skin.

138 **Analysis of anthocyanins by UHPLC-Q-ToF-MS.** Ultra-high-performance liquid chromatography
139 (UHPLC) coupled to quadrupole time-of-flight mass spectrometry (Q-ToF-MS; Waters Corp., Milford, MA,
140 USA) was employed to detect anthocyanins. The HPLC separation was performed at 35 °C on a reversed-phase
141 Acquity UPLC BEH C_{18} analytical column (dimensions: 100 mm × 2.1 mm, particle size: 1.7 μm). The
142 parameters and conditions were set according to Machado et al. (2015) with some modifications as follows:
143 injection volume, 5 μL; solvent flow rate, 0.3 mL/min; mobile phase A, water/formic acid (100:3, v/v); mobile
144 phase B, acetonitrile/formic acid (100:3, v/v); and UV detection wavelength, 530 nm. The following gradients
145 were used: 0−30 min, 0−50% B; 30−35 min, 50−100% B; and 35−37 min, 100−0% B. The compounds were

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146 ionized by an electrospray ionizer operated in a positive mode and were scanned at a scanning range of m/z =
147 50 to m/z = 2000. Other parameters were set as follows: desolvation gas flow, 12 L/min; desolvation temperature,
148 400 °C; cone gas flow, 1.2 L/min; source temperature, 100 °C; capillary voltage, 3.0 kV; cone voltage, 30 V;
149 and collision energy, 25.0 eV. The content of anthocyanins was determined using an external calibration curve
150 prepared using malvidin-3-monoglucoside (Extrasynthese, Lyon, France).

151 **Analysis of non-anthocyanin phenolic compounds by UHPLC-MS.** The analysis of non-anthocyanin
152 compounds was conducted out using a UHPLC coupled to an ESI-triple quadrupole mass spectrometer (Dionex
153 Ultimate 3000; Thermo Fisher Scientific, San Jose, CA, USA). The HPLC separation was performed at 30 °C
154 on a reversed-phase C_{18} analytical column (dimensions: 100 mm × 2.1 mm, particle size: 1.9 μm; Thermo
155 Scientific, Waltham, MA, USA). The operating conditions were set as described by Nováková et al. (2010) with
156 some modifications as follows (Novakova et al. 2010): injection volume, 5 μL; flow rate of mobile phase, 0.3
157 mL/min; mobile phase A, acetonitrile; and mobile phase B, 5 mM ammonium acetate. The gradient elution was
158 conducted as follows: 0−1 min, 0−10% A; 1−3.5 min, 10−40% A; 3.5−5 min, 40−65% A; 5−11 min, 65−90%
159 A; 11− 12 min, 90% A; 12−12.1 min, 90−10% A; and 12.1−14 min, 10% A. The MS was equipped with a
160 negative ion mode ESI and operated at a capillary voltage of 3.0 kV, an ion source temperature of 300 °C, a
161 desolvation temperature of 400 °C, a cone gas flow of 0.2 L/min, a source temperature of 100 °C, a cone voltage
162 of 35 V, and a collision energy of 25.0 eV.

163 **Antioxidant activity assays.** The antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH),
164 which is defined as the amount of an antioxidant required to decrease the initial DPPH concentration by 50%
165 (EC50), was determined as previously described (Katalinić et al. 2010). The EC_{50} was expressed as the gallic
166 acid equivalent. The antioxidant capacity against 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)
167 was determined as previously described (Re et al. 1999). The ferric-reducing antioxidant power (FRAP) was

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168 determined described by Sun et al. (2011). The antioxidant capacity against ABTS and FRAP was expressed as
169 Trolox equivalents.

170 **Wine vinification.** Sixty kilograms of grapes in each replicate were used in small-scale wine making. The
171 winemaking process was conducted following our previously reported method with slight modifications (Yue et
172 al. 2018). First, berries from the CTR treatment and control grapevines were harvested by hand and then
173 squeezed using a squeezing roller to obtain grape musts. After adding sulfur dioxide (50 mg/L), the grape musts
174 were transferred to a 50 L stainless steel fermentation tank and thereafter pectinase was added (20 mg/L). The
175 grape musts were cooled to 5-10 °C within 12 h and then incubated at these temperatures for 48 h. After that,
176 they were heated to 18 °C. After being activated at 39 °C for 30 min, yeast (Lafford F15, 20 mg/L) was added
177 to the grape musts to initiate the alcoholic fermentation. During the fermentation, the temperature was below 22
178 °C on the first day, 25 °C on the second day, and below 28 °C on the third day. The wine cap was punched down
179 twice daily to ensure that it remained submerged in the musts. After 7 days of maceration, the fermentation was
180 concluded when the specific gravity of fermentation liquor dropped to 0.99 g/cm^3, and the residual sugar was
181 below 5 g/L. The wine residue was then pressed, and both the free-run and pressed wines were combined and
182 stored in vessels. Malolactic fermentation was carried out and monitored over a one-month period. After one
183 month, the fermentation was terminated by sulfur dioxide (50 mg/L), and the resultant wines were cold-stabilized
184 at 4 °C for 1 month. The finished wines were filtered, bottled, and stored.

185 **Quantitative real-time PCR (RT-qPCR) analysis.** The level of expression of genes in grape skin in
186 response to CTR was measured using RT-qPCR. Total RNA of the grape skin was extracted using a FastPure
187 Plant Total RNA Isolation Kit (RC401, Vazyme, Nanjing, China), and cDNA was synthesized from the total
188 RNA using a cDNA synthesis kit (HiScript q-RT SuperMix; Vazyme). RT-qPCR was performed using SYBR
189 Green MasterMix (SYBR Premix EX Taq TM, Dalian, China) on a Bio-Rad iQ5 instrument (Hercules, CA).

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Statistical analysis. All the statistical analysis was performed by SPSS software (version 19.0, IBM, Inc., Armonk, NY). The data were subjected to a one-way analysis of variance (ANOVA), and the difference between mean values was determined at 1% or 5% using the Duncan's multiple range test.

Results and Discussion

Effect of CTR of clusters and berry attributes. Representative photographs of CTR of the inflorescences are shown in Figures 1A and E. As a result of CTR, the compactness of the cluster decreased (Figures 1B, C, F, and G), and most berries, including those at the bottom, received more sunlight after CTR. The color of berries inside and at the bottom of the cluster were darker than those of the control (Figures 1D, H, and I).

The cluster weights of pruned clusters harvested in years 2017 and 2018 were 70.66% and 71.63%, respectively, of those of the control. Additionally, the berry number/cluster length and cluster weight/cluster length ratios decreased by 20.53% and 36.86%, respectively, in 2017, and by 34.38% and 39.11%, respectively, in 2018, compared with those of the control. This suggests that the CTR could decrease the compactness. The width and length of pruned berries harvested in 2017 and 2018 were also slightly increased after CTR. However, they were not different from those of the control. The weights of berries harvested in 2017 and 2018 increased by 5.92% and 10.92%, respectively, compared with those of the control (Table 1).

The Brix and skin/berry ratio of the samples also increased after CTR treatment. The Brix in 2017 and 2018 increased by 10.64% and 6% compared with those of the control. The skin/berry ratio of berries harvested in 2018 increased by 15.04% compared with that of the control. The TA of berries harvested in 2017 and 2018 also decreased by 20.28% and 13.4%, respectively, compared with those of the control.

Clusters that are exposed to sunlight (achieved by basal defoliation) generally contain higher contents of sugars, anthocyanins and flavonols, but lower concentrations of malic acid and TA compared with those of shaded clusters (Wang et al. 2018b). The removal of leaves can improve the sunlight exposure of outer berries,

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212 but not the inner ones. Thus, one method for improving fruit quality is to allow the inner berries to sufficiently
213 receive or be more exposed to sunlight. Unlike the traditional method, in which native wine growers usually
214 remove only one cluster from one shoot, the CTR method not only reduces the fruit load, but also increases the
215 sunlight exposure of most berries in a cluster. We found that thee CTR of inflorescence technique could decrease
216 the degree of cluster compactness, as well as the berry number/cluster length and cluster weight/cluster length
217 ratios, because this technique allows the inner berries of clusters to receive more sunlight. Too high a yield has
218 a negative effect on the ripening and quality of grape berries and in turn, can decrease the quality of wine. CTR
219 could remove approximately one-third of berries and reduce the fruit load. Similar to the floral cluster pruning
220 (Zhang et al. 2016), the CTR of inflorescence could increase the berry weight and Brix and decrease the TA. It
221 is likely that the CTR of inflorescence allows the remaining berries to accumulate more photosynthetic products,
222 causing their TSS content to be higher.

223 In addition to the CTR of inflorescences, seasons can also greatly affect the Brix. In 2017, one of the years,
224 in which the study was conducted, had abundant rainfall and low sunlight hours, particularly in July, which is
225 the veraison period. The increase in Brix content in grape berries grown in 2017 was more accentuated than that
226 in grape berries grown in 2018. This observation suggests that the climatic conditions could affect the efficacy
227 of our CTR technique.

228 **Differences between contents of total phenolics, flavonoids, flavanols, anthocyanins and tannins in**
229 **berry skins and wines.** Skin at the distal end of the berries cannot sufficiently receive sunlight owing to cluster
230 compactness that was too high. Thus, we divided the skin of a berry into two parts: the pedicel end and the distal
231 end (Figure S1). As shown in Table 2, the concentrations of total phenolics and total anthocyanins in the pedicel
232 end of the berry skins were higher than those in the distal end. CTR also caused the increase in the concentrations
233 of total phenolics, flavonoids, anthocyanins and tannins in the distal end skins of the berries, as well as in wine
234 prepared from these berries. Additionally, CTR also reduced the difference between the concentrations of these
235 compounds (except for flavonoids) in skins at the two parts of the berries (Table 2). The concentrations of total

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236 phenolics, flavonoids, anthocyanins and condensed tannins in the pedicel end of CTR berries were 7.56%,
237 29.29%, 6.06%, and 9.45%, respectively, which were higher than those of the skins at the same part of the control
238 berries. Similarly, the concentrations of total phenolics, flavonoids, anthocyanins and condensed tannins in the
239 distal end skins of pruned berries were 50.52%, 66.46%, 12.13%, and 31.47%, respectively, which were also
240 higher than those in the skins at the same part of control berries. Nonetheless, the concentrations of total flavanols
241 in the pedicel and distal end skins of the berries were lower compared with those in the skins at the same parts
242 of the control berries.

243 Phenolic compounds primarily accumulate in berry skins. According to Río Segade et al. (2008), berry skin
244 thickness is an index that can be used to efficiently predict the extractability of anthocyanins. A previous study
245 showed that mild to moderate water deficit and early leaf removal technique can result in a higher berry skin/flesh
246 ratio (Zsófi et al. 2014). In this study, we found that the CTR of inflorescence increased the skin/berry ratio,
247 which may be owing to the increased sunlight exposure of the distal end of both inner and outer berries.
248 Therefore, the increased thickness of berry skin caused by CTR could increase the sink capacity and result in
249 higher concentration of phenolic compounds. In addition, the impact of CTR on phenolic compounds could be
250 the result of changes in microclimates, which cause the metabolic pathway to be promoted. A previous study
251 has shown that sunlight exposure has a direct effect on the metabolic pathway, causing the increase in synthesis
252 of some phenolic compounds (Boss and Davies, 2009).

253 It is worth discussing that while the CTR increased the sunlight exposure on berry skins, it also reduced the
254 cluster load, A previous study showed that although cluster thinning technique has a positive effect on the quality
255 of berries, it would also enlarge the berry size and weight in the clusters retained (Gil et al. 2013, Diago et al.
256 2010), thereby reducing the concentrations of phenolics and anthocyanins (mg/g). However, in this study, the
257 berry weight and berry composition of CTR berries were both greater than those of the control berries in 2018
258 (Table 1), which suggested that the increase in berry composition (phenols and anthocyanins should be attributed
259 more to the sunlight exposure rather than the reduced load. This was further proven by the differences between

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260 two parts berry skins, in terms of berry composition, such as flavonoids, anthocyanins and condensed tannins,
261 the distal end berry skins had more obvious improvements than the pedicel end berry skins under CTR treatment
262 (Table 2).

263 **CTR on inflorescence increased the antioxidant activities of berry skins and wines.** Because there is
264 no universal assay that can reflect all the antioxidant activities of a complex system, we employed three assays
265 to evaluate the antioxidant activities of grape skins and wines (Table 3). The results of FRAP and ABTS assays
266 showed that the antioxidant activities of pedicel end of control berries were much higher than those of distal end
267 skins of berries. The skins of CTR berries exhibited a much higher antioxidant capacity than the skins of control
268 berries, particularly for those at the distal end. The concentrations of FRAP and ABTS in the pedicel end of
269 berries were 30.95% and 18.02%, respectively, higher than those in the skins at the same part of control berries.
270 In addition, the contents of FRAP and ABTS in the distal end skins of berries were 30.95% and 18.02%,
271 respectively, higher than those of the skins at the same part of control berries. In addition, the contents of DPPH
272 and FRAP in wines prepared from CTR berries were 31.4% and 33.70%, respectively, higher than those in wines
273 prepared from the control berries.

274 Furthermore, the Pearson's correlation between the antioxidant activity and the phenols content was
275 determined. A weak correlation between the concentrations of phenols and the DPPH was observed in all
276 samples, which indicates that DPPH is not sensitive to the antioxidants in grape berries (Table 3). The correlation
277 of total phenols, flavonoids, flavanols and anthocyanins with FRAP and ABTS were significant, and there was
278 a strong correlation between total anthocyanins and ABTS ($P < 0.01$).

279 SET methods (single electron transfer), such as ABTS, DPPH and FRAP, are based on the determination
280 of REDOX potential and are widely used because of their simple operation and high sensitivity. DPPH is a
281 synthetic and stable free radical, which presents a deep purple color and a strong absorption band in the range of
282 515–520 nm. In the presence of antioxidant compounds, DPPH can accept an electron or a hydrogen atom from

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283 the antioxidant scavenger molecule to be converted to a more stable DPPH molecule. Since the reduced form of
284 DPPH is pale yellow, it is possible to determine the antioxidant activity by measuring the change in color
285 spectrophotometrically (Yolanda et al. 2014, Molyneux, 2004). The ABTS assay is also based on decoloration,
286 which occurs when the radical cation $ABTS^+$ is reduced to ABTS (Re et al. 1999, Katalinić et al. 2010). The
287 antioxidant activity of polyphenols is related to the number and location of phenolic hydroxyl groups. The higher
288 the number of phenolic hydroxyl groups, the stronger the antioxidant activities. Therefore, many biologically
289 active compounds, including anthocyanins and other antioxidant phenolic compounds, play a key role in
290 preventing oxidative damage (Wang et al. 2018a, Fang et al. 2019, Sun et al. 2019). In this study, we observed
291 that there was a strong correlation between the activities of ABTS and FRAP and anthocyanins, which suggests
292 that anthocyanins are the primary contributors to the antioxidant activities of wines. One previous study has also
293 reported similar results (Meng et al. 2012).

294 **CTR of inflorescence increased the content of anthocyanins in berry skins.** The skins of berries in CTR
295 grapevine contained higher concentrations of anthocyanins than those of berries in the control grapevine, and
296 these anthocyanins primarily included dephinidin-3-O-glucoside (Dp), petunidin-3-O-glucoside (Pt), malvidin-
297 3-O-(trans-6-o-coumaryl)-glucoside (t-Mv-co) and malvidin-3-O-glucoside (Mv) (Table 4). Whereas Mv and its
298 derivative, t-Mv-co, occupied the largest proportion of anthocyanins, cyanidin was undetectable in the skins of
299 berries in both the control and CTR grapevines. Moreover, as a result of CTR, the concentrations of Pt, Dp, Mv
300 and t-Mv-co in berry skins increased by 229.62%, 108.93%, 98.49% and 52.42%, respectively. Additionally,
301 compared with that in berries from the control grapevine, the concentration of total anthocyanins, as well as of
302 t-Mv and t-Mv-co, in berries from CTR grapevine also increased. A previous study reported that the contents of
303 Mv derivatives are the highest among all 33 different types of anthocyanins (Liang et al. 2008). It is likely that
304 Mv and its derivatives are the main contributors to the antioxidant activities of grape berries and wines.

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305 **Composition and concentration of non-anthocyanin phenolic compounds in control and CTR berries.**
306 The concentrations of non-anthocyanin phenolic compounds in the CTR berry skins were higher than those in
307 the control, likely owing to the presence of higher concentrations of non-flavonoid phenolics and flavonols (not
308 flavanols) (Table 5). *p*-Coumaric acid was the most abundant non-flavonoid phenolic compound in the berry
309 skin. Furthermore, the concentrations of caffeic acid, *p*-coumaric acid and gallic acid in the skins of berries from
310 CTR grapevine were 295.53%, 45.42%, and 33.10%, respectively, higher than those in the control berries,
311 particularly for those in the skins at the pedicel end part of the berries. In addition, the change in flavonols was
312 the same as that in non-flavonoid compounds, and isoquercitroside was the most abundant flavonol compound,
313 accounting for 85.3% and 89.8% in the skins at the distal end and pedicel end, respectively, of berries from CTR
314 grapevines. It is worth mentioning that the increments of these flavonol compounds in the distal end skins of
315 berries were more obvious than those in the pedicel end. The concentrations of naringin and isoquercitroside in
316 the distal end skin of berries from CTR grapevine were 232.7% and 56.73%, respectively, higher than those in
317 the same part of berries from control grapevine. Additionally, the concentrations of naringin and isoquercitroside
318 in the pedicel end of berries from CTR grapevine were 74.32% and 15.37%, respectively, higher than those in
319 the skins of berries from control grapevines. Kaempferol was only detected in the skins (both the pedicel end
320 and distal end) of berries from CTR grapevine. In contrast, the concentration of (+)-catechin in berries from CTR
321 grapevine was lower than that in berries from control (unpruned) grapevine. As the main contributor of flavanols,
322 the concentrations of (+)-catechin in the pedicel end and distal end berry skins were 83.3% and 82.18%,
323 respectively, lower than those in the control.

324 Sunlight exposure is important factor that causes the accumulation of phenolic compounds in grapes (Wang
325 et al. 2018b), and early leaf removal can have a higher effect on the accumulation of phenolic composition than
326 cluster thinning (Bubola et al. 2017). Compared with the above two techniques, CTR not only increased the
327 sunlight exposure of the clusters, but also decreased activity per grapevine. After CTR, the increase in
328 concentrations of gallic acid, *p*-coumaric acid and caffeic acid were the most prominent among all non-flavonoid

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329 phenolic compounds, and the increase in concentrations of kaempferol and myricetin were the most prominent
330 among all flavonol compounds. Increasing sunlight exposure can obviously promote the synthesis of flavonols
331 in grapes (Downey et al. 2008). Another study on Malbec grape has also suggested that cluster thinning can
332 enhance the synthesis of dihydroquercetin-3-glucoside and other flavonol compounds (Martín et al. 2011).

333 **Effect of CTR on relative expressions of genes involving in phenolic biosynthesis.** To understand the
334 effect of CTR on the accumulation of phenolic compounds at the transcriptional level, several relevant genes
335 involving in phenolic biosynthesis were analyzed using RT-qPCR (Figure 2). The analysis showed that the levels
336 of expression of *VvFLS1* (flavonol synthase/flavanone 3-hydroxylase 1), *VvF3'H* (flavonoid 3'-hydroxylase),
337 *VvF3'5'H* (flavonoid-3' 5'-hydroxylase), *VvANS* (anthocyanidin synthase) and *VvMYBA1* in the distal end skins
338 of berries from CTR grapevine were much higher than those in the distal end skins of berries from control
339 grapevine. In particular, the most obvious change was observed in *VvFLS1*. The expression of this gene in the
340 skins of berries from CTR grapevine was 23.06-fold higher than that in the skins of berries from control
341 grapevine. Moreover, the expression of *VvMYBA1* in the skins of berries from CTR grapevine was upregulated
342 to 12.25-fold higher than that in the skins of berries from control grapevine. However, compared with that of
343 other genes, the level of expression of *VvLAR1* (leucoanthocyanidin reductase 1) had the opposite trend.

344 Changes in phenolic compounds could be attributed to changes in the profiles of expression of related
345 genes. A previous study has shown that sunlight exclusion has a negative effect on related structural and
346 regulatory genes, including chalcone synthase (*CHS*), chalcone isomerase (*CHI*), *F3'H*, dihyoflavonol reductase
347 (*DFR*), leucoanthocyanidin dioxygenase (*LDOX*), *UFGT* and *MYBA1* in the berry skin of Cabernet Sauvignon
348 (Jeong et al. 2004). One previous study reported that during seedlings of Lambrusco grape, the contents of *CHS*,
349 *CHI*, *F3H*, *DFR*, *LDOX* and *UFGT* transcripts were very low when the grape was grown in darkness but were
350 dramatically increased when the grape was exposed to 6 h of light (Sparvoli et al. 1994). In this study, the
351 upregulation of *VvF3'H* and *VvF3'5'H* may increase the production of dihydroquercetin and dihydromyricetin,
352 which could be then catalyzed to quercetin and myricetin by *VvFLS1*, of which the gene expression in the skins

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of berries from CTR grapevine was 23.06-fold higher than that of the skins of berries from control grapevine. After that, leucocyanidin may be generated and converted to (+)-catechin, as indicated by the level of expression of *VvLAR1* was consistent with the accumulation of (+)-catechin. In addition, Mv could be synthesized downstream, and the expression of *VvMYBA1* can benefit this step. The expression of *VvMYBA1* gene, the key gene that can regulate the coloration of berry skin (Walker et al. 2007), in the skins of berries from CTR grapevine was 9.58-fold higher than that in the skins of berries from control group. The elevated expression of *VvMYBA1* and *VvANS* were consistent with the accumulation of anthocyanins in the skins of berries from CTR grapevine. Based on the above observation, it appears that *VvMYBA1* may participate in the metabolic regulation of anthocyanins and other phenolic compounds by regulating the expression of genes involving in phenolic acid biosynthesis.

Conclusions

Taken together, we can conclude that the CTR of inflorescence decreased the compactness of the grape cluster and increased Brix and skin/berry ratio. Berries from CTR grapevine and wine prepared from these berries contained higher concentrations of total anthocyanins, total phenols, and flavonoids, as well as higher antioxidant activities, compared with those in berries from control grapevine. We also observed a correlation between the concentrations of anthocyanins and the antioxidant activity. The CTR was found to increase the concentrations of anthocyanin derivatives, including Dp, Pt, t-Mv-co and Mv. It also caused the increase of concentrations of non-anthocyanin phenolic compounds, including gallic acid, *p*-coumaric acid, caffeic acid, naringin, kaempferol, isoquercitroside, myricetin and (+)-catechin. Lastly, we found that the CTR upregulated the expression of *VvFLS1*, *VvF3'H*, *VvF3'5'H*, *VvANS*, and *VvMYBA1* but downregulated the expression of *VvLAR1* gene.

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Table 1 Yield and other properties of Marselan berries in Great Wall vineyard in Penglai (Shandong, China) from 2017 to 2018.

	2017		2018	
	Control	CTR	Control	CTR
Yield ton/ha	9.15 ± 0.32	6.18 ± 0.27**	9.65 ± 0.38	6.10 ± 0.41**
Cluster weight (g)	197.81 ± 26.97	139.77 ± 22.19**	296.7 ± 34.25	212.53 ± 23.63*
Berry weight (g/100 berries)	122.03 ± 13.46	129.25 ± 10.96	139.2 ± 12.48	154.4 ± 9.69*
Mean berries per cluster	169.00 ± 14.18	116.67 ± 16.17**	213.67 ± 11.24	144.33 ± 19.22**
Berry number/cluster length	12.32 ± 0.28	9.79 ± 0.77**	10.15 ± 0.34	6.66 ± 0.25**
Cluster weight/cluster length	14.16 ± 0.24	8.94 ± 0.52**	12.07 ± 0.14	7.35 ± 0.39**
Berry width (mm)	13.89 ± 1.12	14.71 ± 0.87	14.69 ± 1.33	15.03 ± 0.69
Berry length (mm)	11.94 ± 1.43	12.26 ± 1.26	12.14 ± 1.32	13.88 ± 0.79
Skin/berry ratio (%)	_	_	15.49	17.82*
Brix (%)	19.07 ± 0.28	21.1 ± 0.35*	23.33 ± 0.42	24.73 ± 0.64*
pH	4.13 ± 0.01	4.17 ± 0.01**	4.13 ± 0.03	4.14 ± 0.05
Titratable acidity (g tartaric acid/L)	4.88 ± 0.04	3.89 ± 0.09**	3.88 ± 0.05	3.36 ± 0.08**

CTR: cluster tip removal. Values are expressed as the means ± SD of three replicates. * $p < 0.05$. ** $p < 0.01$.

Table 2 Concentrations of total phenolics, flavonoids, flavanols, anthocyanins and tannins in Marselan berry skins and wines from Great Wall vineyard in Penglai (Shandong, China) in 2018.

	Pedicel end		Distal end		Wine	
	Control	CTR	Control	CTR	Control	CTR
Total phenolics	39.54 ± 2.30	42.53 ± 2.74	24.78 ± 0.77	37.30 ± 2.76*	1.76 ± 0.04	1.87 ± 0.02
Total flavonoids	19.56 ± 0.68	25.29 ± 0.78*	19.62 ± 1.89	32.66 ± 0.76*	0.79 ± 0.02	0.83 ± 0.03
Total flavanols	9.68 ± 0.14	8.26 ± 0.11**	6.26 ± 0.3	5.02 ± 0.11**	0.26 ± 0.01	0.30 ± 0.01**
Total anthocyanins	16.18 ± 1.37	17.16 ± 0.39	12.70 ± 0.55	14.24 ± 0.38**	0.17 ± 0.01	0.32 ± 0.01**
Condensed tannins	13.12 ± 1.06	14.36 ± 0.96	9.85 ± 0.33	12.95 ± 0.92**	1.38 ± 0.04	1.50 ± 0.03

The skin of one berry was divided to two types: the pedicel end and the distal end. CTR: cluster tip removal. Values are means ± SD of three replicates. * $p < 0.05$. ** $p < 0.01$. The units of these parameters are mg/g FW or /mL wine.

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Table 3 Antioxidant activities of Marselan berry skins and wines from Great Wall vineyard in Penglai (Shandong, China) in 2018.

		DPPH	FRAP	ABTS
Pedicel end	Control	0.098 ± 0.005	56.02 ± 1.43	14.76 ± 0.14
	CTR	0.0929 ± 0.004	73.36 ± 0.44**	17.42 ± 0.39**
Distal end	Control	0.074 ± 0.009	37.44 ± 1.51	12.35 ± 0.25
	CTR	0.092 ± 0.012	68.54 ± 1.36**	19.18 ± 0.45*
Wine	Control	0.105 ± 0.002	144.14 ± 5.15	49.44 ± 1.00
	CTR	0.138 ± 0.003**	192.72 ± 3.03**	52.83 ± 0.87
Pearson correlation coefficient	Total phenols	0.625	0.833*	0.908*
	Total flavonoids	0.687	0.824*	0.878*
	Total flavanols	0.613	0.830*	0.892*
	Total anthocyanins	0.703	0.894*	0.960**

DPPH represents DPPH free radical-scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl. FRAP represents ferric ion reducing antioxidant power. ABTS represents 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid). The DPPH assay was provided as $1/EC_{50}$, the unit of EC_{50} is mg gallic acid equivalent /g FW. The units of ABTS and FRAP are mg Trolox/g FW. The skin of one berry was divided to two types: the pedicel end and the distal end. CTR: cluster tip removal. Values are reported as the means ± SD of three replicates. * $p < 0.05$. ** $p < 0.01$.

Table 4 Species and concentrations of anthocyanins in Marselan berry skins from Great Wall vineyard in Penglai (Shandong, China) in 2018.

Component	Control (μg/g FW)	CTR (μg/g FW)
Pt-co	1.94 ± 0.28	2.40 ± 0.21
Dp	2.31 ± 0.15	4.82 ± 0.17*
Pt	2.87 ± 0.14	9.47 ± 0.27*
t-Mv-co	31.02 ± 3.58	47.28 ± 3.62*
Mv	90.27 ± 5.41	179.17 ± 6.82*

Pt-co: petunidin-3-*O*-(6-*O*-coumaryl)-glucoside. Dp: dephinidin-3-*O*-glucoside. Pt: petunidin-3-*O*-glucoside. t-Mv-co: malvidin-3-*O*-(trans-6-*O*-coumaryl)-glucoside. Mv: malvidin-3-*O*-glucoside. CTR: cluster tip removal. Values are means ± SD of three replicates. * $p < 0.05$.

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Table 5 Concentrations of different non-anthocyanin phenolic compounds in Marselan berry skins from Great Wall vineyard in Penglai (Shandong, China) in 2018. (Unit: mg/kg FW).

	Distal end		Pedicel end	
	Control	CTR	Control	CTR
Non-flavonoid phenolic compounds				
Gallic acid	196.29 ± 18.21	307.83 ± 2.41**	236.65 ± 5.63	314.97 ± 3.07**
p-Coumaric acid	505.64 ± 4.56	591.36 ± 0.765**	416.73 ± 9.18	606.011 ± 2.64**
Caffeic acid	110.99 ± 10.30	101.47 ± 2.63	41.64 ± 2.94	164.70 ± 22.43**
SUM	812.92	1000.66	695.02	1085.681
Flavonols compounds				
Naringin	44.28 ± 1.68	147.32 ± 13.67**	43.62 ± 5.94	76.04 ± 2.60**
Kaempferol	-	147.12 ± 5.83**	-	85.09 ± 11.58**
Isoquercitroside	2696.35 ± 8.65	4225.97 ± 394.86**	5666.35 ± 7.91	6537.49 ± 209.06*
Myricetin	312.37 ± 28.98	435.92 ± 1.01**	510.51 ± 4.91	583.23 ± 26.24**
SUM	3053	4956.33	6220.48	7281.85
Flavanols compounds				
(+)-Catechin	103.42 ± 0.33	17.24 ± 1.60**	150.22 ± 20.46	26.77 ± 0.59**
(–)-Epigallocatechin	124.86 ± 0.40	115.17 ± 10.69	145.26 ± 19.78	121.06 ± 3.69
SUM	228.28	132.41	295.48	147.83

The skin of one berry was divided to two types: the pedicel end and the distal end. CTR: cluster tip removal. Values are means ± SD of three replicates. * $p < 0.05$. ** $p < 0.01$. "–" not detected.

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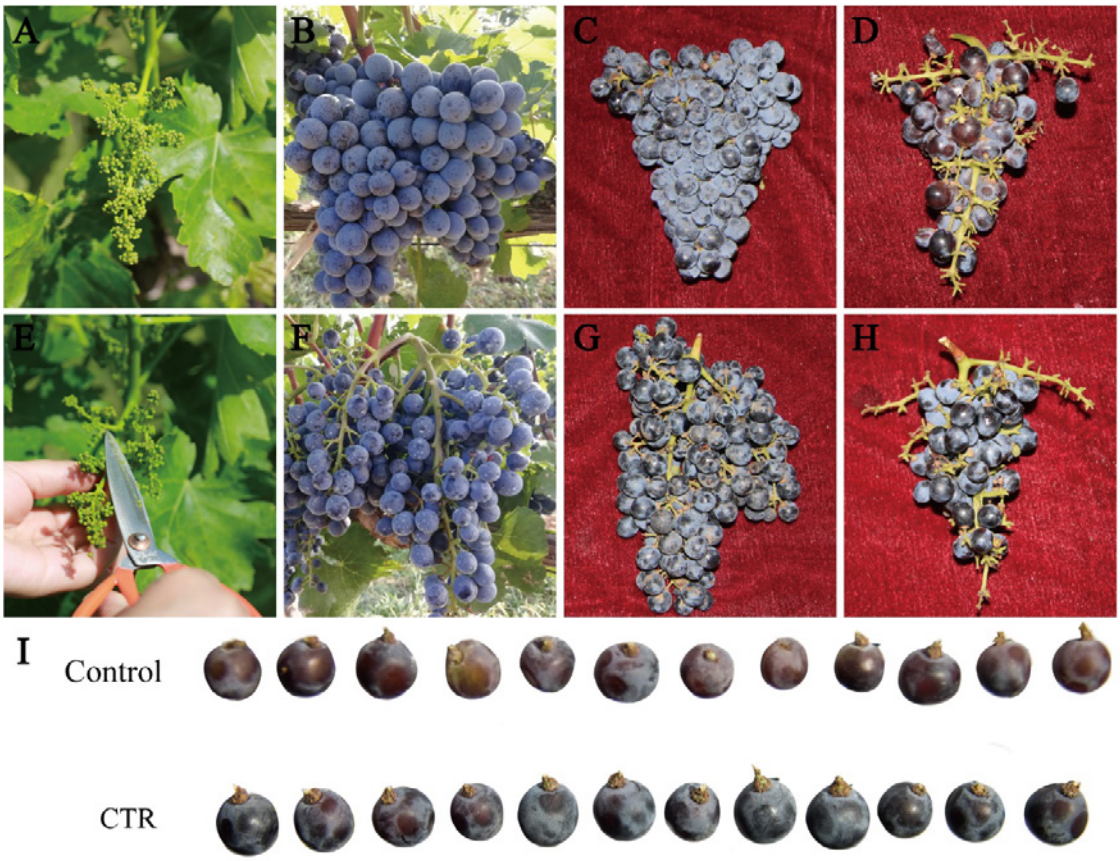

Figure 1 Comparison of cluster compactness and skin color of berries in the cluster before and after CTR (cluster tip removal) of Marselan inflorescences from Great Wall vineyard in Penglai (Shandong, China). (**A**) Control inflorescence. (**B**) and (**C**) Control cluster. (**D**) Inner berries from control cluster. (**E**) Demonstration of the CTR of inflorescences. (**F**) and (**G**) Cluster of inflorescences obtained after CTR. (H) Inner berries from CTR grapevine. (I) Inner berries at the bottom of a cluster from control grapevine (Top) and from CTR grapevine (Bottom).

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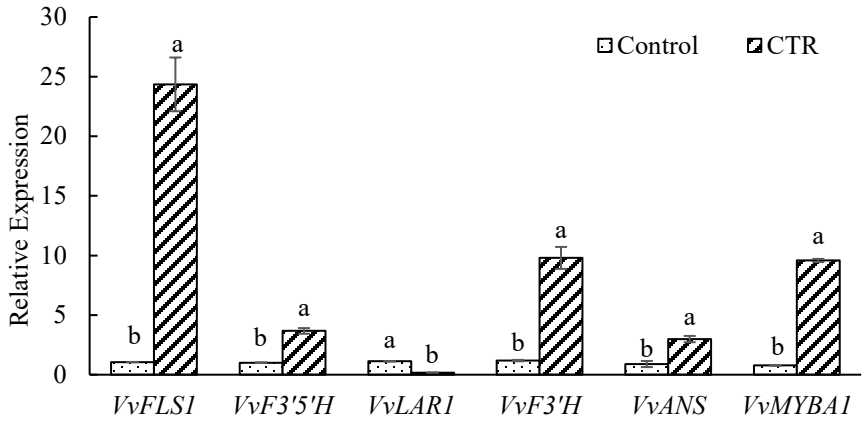

Figure 2 Transcriptional changes of the phenolic biosynthesis-related genes in grape berry skins by CTR. Relative levels of expression, determined by RT-qPCR, of *VvFLS1*, *VvF3'H*, *VvLAR1*, *VvANS*, *VvMYBA1* and *VvF3'5'H* genes in distal end of berry skins as a result of CTR. *VvActin* was used as an internal control to which each gene expression was normalized. CTR: cluster tip removal. *VvFLS1:* flavonol synthase. *VvF3'H:* flavonoid 3′-hydroxylase. *VvLAR1:* leucoanthocyanidin reductase. *VvANS:* anthocyanin synthase. *VvF3'5'H:* flavonoid 3′,5′-hydroxylase. Different lowercase letters indicate significant differences ($p < 0.05$).

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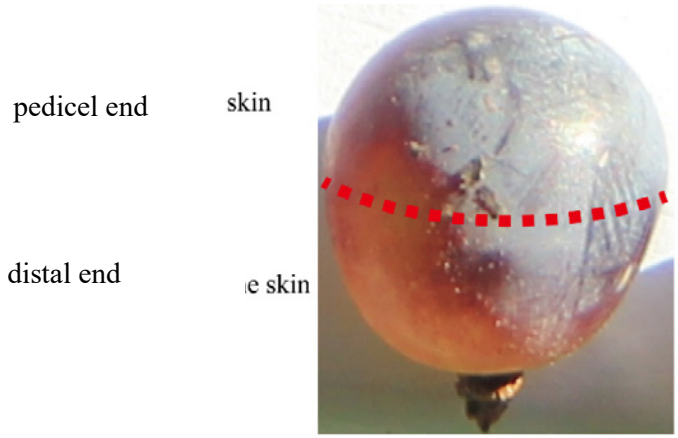
Supplemental Table 1 Daily average temperature, sunlight hours and rainfall in Penglai during 2017 to 2018.

Climatic conditions	Year	April	May	June	July	August	September
Daily average temperature (°C)	2017	14.78	21.37	24.08	27.88	26.61	23.21
	2018	15.62	20.56	25.72	26.71	27.08	21.31
Sunlight hours (h)	2017	253.7	327.4	286.5	168.7	185.3	245.2
	2018	253.5	261.1	257.2	247.7	261	211.7
Rainfall (mm)	2017	13.2	9	22	156.8	190.2	10.2
	2018	46.6	60.6	55.2	55	100.4	4.8



Supplemental Figure 1 The skin of one berry was divided to two types: the pedicel end and the distal end.