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RELATIONSHIP BETWEEN 2-PHENYLETHANOL CONTENT AND DIFFERENTIAL EXPRESSION OF L-AMINO ACID DECARBOXYLASES (AADC) IN (*VITIS VINIFERA*) VIDAL WINE GRAPE AT DIFFERENT LOADS

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Abstract

In this study, the headspace-solid phase microextraction-gas chromatography/mass spectrometry (HS-SPME-GC/MS) was used to determine the type and content of aroma in Vidal grapes. A quantitative fluorescence measurement was performed to determine the differential expression of Amino Acid Decarboxylase (AADC). By conducting five different load treatments (fruit weight per 667 m^2: 750, 1,000, 1,250, 1,500, and over 1,750 kg), we found that the main components of Vidal grapes were alcohols, esters, alkanes, aldehydes, phenols, ketones, and ethers. The relative levels of alcohols, esters, alkanes, and phenols were 25, 27, 18, and 14%, respectively. The relationship between the dynamic content of the characteristic aroma component 2-phenylethanol and the expression of AADC enzyme was explored. The results showed that for a small load, the relative expression levels of 2-phenylethanol-regulating AADC enzyme were high and low in the early and late stages of growth, respectively. For a large load, the content of 2-phenylethanol was low, while the relative expression levels of 2-phenylethanol-regulating AADC enzyme were low and high in the early and late stages of growth, respectively. In the early stage, the positive regulation was significant, and in the late stage, the relative expression of AADC was increased rapidly, which in turn, increased the positive regulation. It was recommended that the suitable yield for Vidal grape during peak fruiting period was 1,000~1,500 kg per 667 m^2. This study provides the scientific basis for the control of fruit aroma and can be used as a reference for load adjustment in the production of wine grape during peak fruiting period.

Key words: Grape; Load; 'Vidal'; 2-phenylethanol; Aromatic L-amino acid decarboxylase (AADC).

Introduction

Fruits are the main sink organs of fruit trees (Pavel & Dejong, 2006) and the load of a fruit tree has a very important impact on the growth and development of a tree (Wu *et al.*, 2011; Shi *et al.*, 2012). The load of fruit trees has a relatively high impact on the photosynthetic rate, but the extent of impact varies. Some studies have shown that the higher the load of a fruit tree, the higher its net photosynthetic rate (Palliotti *et al.*, 2000; Bogicevic *et al.*, 2015; Li *et al.*, 2015). Some studies have indicated that the higher the load of a fruit tree, the lower its net photosynthetic rate (Kaps *et al.*, 1989; Iglesias *et al.*, 2002; Uriarte *et al.*, 2016). Other studies have found that the level of an adult citrus tree load has no impact on the net photosynthetic rate (Fu *et al.*, 2011). However, the load of a fruit tree has a significant effect on its fruit quality.

Ice wine was originated in the Franken region of Germany at the end of the 17th century. Because of the constraint of natural weather conditions, only some regions in Canada, Germany, Austria, China, and a few other countries could produce ice wine grapes in natural conditions. The ice wine grape production of Liaoning Province of China in 2012 accounted for approximately 47% of the world's total output of ice wine. However, different years, origins, sample treatment methods, and detection methods can cause the differences in aroma components (Zhao *et al.*, 2014). Yang *et al.*, (2011) found that the synthesis and the change in content of the characteristic aroma component 2-phenylethanol in Vidal grape was related to a rapid temperature drop in the production area. In 2009, Wang *et al.*, (2014) detected a high content of furfural in the grape juice prepared from the fruits harvested during the maturation and freezing periods in Jilin, Tonghua. In 2010, Ma (2015) used a combination of gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry (GC-MS) and other advanced detection measures to isolate 63 aroma-active compounds in Vidal ice wine, and determined that β-damascenone was the key aroma compound in the ice wine and 3-(methylthio) propanal and 4-hydroxy-2,5-dimethyl-3(2H)-furanone also had a significant effect on the aroma perception of ice wine. Tian *et al*., (2012) studied the effect of three different rootstocks on the quality of mature fruits and found that 'Tianzhen 2' could improve the fruit quality, whereas the other two rootstocks reduced the related quality. On one hand, grafting affects the fruit quality and activity of enzymes related to aroma synthesis by delaying fruit ripening; on the other hand, the level of transcription affects the expression level of the enzymes related to aroma synthesis, ultimately affecting the composition and quality of fruit aroma.

In order to study the effects of different fruit loads on the dynamic change in the content of 2-phenylethanol and the differential expression of AADC enzyme in wine grape 'Vidal' in the semi-arid area of western Liaoning, this experiment was conducted from June 2015 to July 2016. The relationship between the dynamic content of 2-phenylethanol in wine grape 'Vidal' and the differential expression of AADC enzyme was explored. The mechanism of the formation and regulation of 2-phenylethanol in wine grape 'Vidal' was studied in order to provide the theoretical basis for the suitable fruit load for the production of high-quality raw materials for ice wine.

Materials and Methods

The 10-year-old wine grape (*Vitis vinifera*) variety 'Vidal' was used as the experimental material. The seedlings were grafted onto Beta rootstock, the plant spacing was 3.0 × 0.5 m, and the grapevines were planted in a north-south direction. A small trellis was used as a growing rack; independent long-stem pruning was performed, and other field managements were consistent (Lin 2010). We used five load treatments, and the fruit weights per 667 m^2 were 750 kg (treatment 1), 1,000 kg (treatment 2), 1,250 kg (treatment 3), 1,500 kg (treatment 4), and over 1 750 kg (treatment 5). According to the yields of previous years, the average weight of a single cluster was 200 g, and the five load treatments corresponded to 9~10, 12~13, 15~16, 18~20 clusters/vine, and no cluster treatment. Flower thinning was performed between three and five days before flowering; double inflorescences remained on the branches strong in vigor, single inflorescence remained on the branches medium in vigor, and no inflorescence remained on the branches weak in vigor. The diameter of the branch at position of the first inflorescence was used as the criteria: the diameter of the branches strong in vigor was ≥0.8 cm, the diameter of the branches medium in vigor was between 0.5 and 0.8 cm, and the diameter of the branches weak in vigor was ≤ 0.5 cm. Single plant was set as one small area, and six replicates were used.

Quantitative fluorescence measurement

Extraction of total RNA from grape fruit: The total RNA of the grape fruits was extracted based on the modified cetyltrimethylammonium bromide (CTAB) method, and the method improvements were the use of polyvinylpolypyrrolidone (PVPP) and the change of the subsequent phenolic extraction reagent.

Primer design: According to the three gene sequences of tomato AADC published in the National Center for Biotechnology Information (NCBI) GenBank database (DQ124868) (accession numbers: DQ458998, DQ458999, and DQ459000), the primers were designed by *in silico* cloning:

Upstream primer of AADC gene fragment: 5′-GTGTTGGCTAGGTATCGG-3′
Downstream primer of AADC gene fragment: 5′-CCTGTTCCAGAGTGTCCC-3′

Specific polymerase chain reaction (PCR) primers designed for grape *β-actin* sequence were as follows:

Upstream primer: 5′-TACAATTCCATGAAGTGTGATG-3′
Downstream primer: 5′-TTAGAAGCACTTCCTGTCAA CTATG-3′

Real-time quantitative reverse transcription (RT)-PCR

Real-time quantitative RT-PCR reactions were performed using the primers and probes of AADC and actin. The volume of RT reaction system was 10 μl. First, the total RNA, RT primer, and deoxynucleoside triphosphate (dNTPs) were added to a 0.2-ml reaction tube, and the mixture was mixed evenly followed by a denaturation at 65°C for 5 min. Subsequently, the sample was quickly put into the ice-water mixture to cool down, followed by the addition of avian myeloblastosis virus (AMV) reverse transcriptase, reverse transcriptase buffer, RNase inhibitors, etc. The reverse transcription reaction was carried out in a thermal cycler programmed as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 1 min, followed by a final extension step at 72°C for 5 min and storage at 4°C until required for analysis.

Quantitative PCR was performed in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, California, USA) using a 96-well plate covered with an optical film. The amplification reaction was performed in a 10-μl reaction volume, including 1/3 μl RNA (RNA content: 100 ng), 2/3 μl each of forward and reverse PCR primers, 4.5 μl of ultrapure water, and 5 μl of SYBR green dye, and each reaction was repeated three times. The following reaction conditions were used: 40 cycles of 2 min at 50°C, 10 min at 95°C, 10 s at 95°C, 1 min at 60°C, followed by 1 min at 60°C and 15 s at 95°C. The relative expression level was calculated by $2^{-\Delta\Delta Ct}$ method.

Results

Aroma component content analysis of the Vidal fruit: By the technology of gas-chromatography and mass spectrum combined with NIST11spectrum, the aroma component types were detected and divided into 50 kinds of alcohols, esters, alkanes, aldehydes, phenols, acids, ketones and others in total. Alcohols covers 2-Penten-1-ol, 2-methyl-, acetate, geraniol, methanol, 2-phenylethyl alcohol with content of 0.122, 0.0076, 0.1261, 0.0084 and 0.1553 respectively (Table 1).

Esters mainly included methyl salicylate, 3,7-dimethyl-2,6-octadienyl ester, methyl hexadecanoate, geranyl acetate, dimethyl phthalate, pentadecyl methacrylate, diethyl phthalate, methyl ester, diisobutyl phthalate, ethyl α-[β-(diethylamino)ethyl] acetoacetate and dibutyl phthalate. The relative contents were 0.0313, 0.0157, 0.0039, 0.0073, 0.0065, 0.0069, 0.0088, 0.0016, 0.0398, 0.0034 and 0.0316 respectively.

Alkane mainly included hexaethyl cyclotrisiloxane, decamethyl cyclopentasiloxane, hexam ethylcyclotrisiloxane, dodecamethylcyclohexasiloxane, 6, 6-dimethyl octadecane, silane, decane, phenylmethy, cyclononasiloxane octadecamethyl, tetracosane, pentacosane, tris (pentyloxy) silane, octamethyl-3, 5, 5-trimethylsiloxy tetrasiloxane, hexacosane and triacontane. The relative contents were 0.0411, 0.1222, 0.0202, 0.0821, 0.0067, 0.0459, 0.0049, 0.0076, 0.0211, 0.0099, 0.012, 0.0027, 0.003, 0.0133 and 0.0036 respectively.

Phenolic mainly included dimethylphenol, phenol and dibutylphenol. The relative contents 0.0093, 0.0151 and 0.007 respectively.

Acids mainly included caprylic acid with relative content of 0.0433.

Table 1. Aroma component types and content of grape Vidal fruit.

Types	Component	Vidal	Types	Component	Vidal
Alcohols	2-phenylethanol	0.1553	Alkanes	hexaethyl cyclotrisiloxane	0.0411
	2-Penten-1-ol	0.0122		icosane	-
	2,2,6-Trimethyl-6-vinyltetranhydro-2H-pyran-3-ol	0.0076		decamethyl cyclopentasiloxane	0.1222
				hexamethyl cyclotrisiloxane	0.0202
				dodecamethyl cyclohexasiloxane	0.0821
Esters	geraniol	0.1261		6,6- dimethyl octadecane	0.0067
	methanol	0.0084		silane	0.0459
	benzyl alcohol	-		decane	0.0049
	methyl salicylate	0.0313		phenylmethy	0.0076
	3,7-dimethyl-2,6-octadienyl ester	0.0157		cyclononasiloxane octadecamethyl	0.0211
	methyl hexadecanoate	0.0039		tetracosane	0.0099
	geranyl acetate	0.0073		pentacosane	0.012
	dimethyl phthalate	0.0065		tris (pentyloxy) silane	0.0027
	pentadecyl methacrylate	0.0069		octamethyl-3,5,5-trimethylsiloxy tetrasiloxane	0.003
	diethyl phthalate	0.0088		hexacosane	0.0133
	methyl ester	0.0016		triacontane	0.0036
	diisobutyl phthalate	0.0398			
	ethyl α-[β-(diethylamino)ethyl] acetoacetate	0.0034			
	dibutyl phthalate	0.0316			
	dimethylphenol	0.0093		formylglycine	0.0156
Phenols	phenol	0.0151	Others	benethamine	0.0044
	dibutylphenol	0.007		9,9-dimethyl-9-silicon	0.0092
Acids aldehydes ketones	caprylic acid	0.0433		fluoreneacetyl methyl phosphate	0.0095
	benzaldehyde	0.0246		naphthalene	0.0078
	citral	0.0323		eugenol phenylethyl ether	0.0083
	5-hydroxymethylfurfural	0.0042		pyrazoline	0.0042
	geranylacetone	0.0165		3-silylpropylene	0.0039
	acetone	0.0038		oxime	0.0048
	tetrahydro-4H-pyran-4-one	0.0038			

Aldehydes mainly included benzaldehyde, citral and 5-hydroxymethylfurfural with relative contents of 0.0246, 0.0323 and 0.0042 respectively.

Ketone mainly included geranylacetone, acetone and tetrahydro-4H-pyran-4-one. The relative contents were 0.0165, 0.0038 and 0.0038 respectively.

Except for what mentioned above, there were formylglycine, benethamine, 9, 9-dimethyl-9-silicon fluoreneacetyl methyl phosphate, naphthalene, eugenol phenylethyl ether, pyrazoline, 3-silylpropylene and oxime, whose relative contents were 0.0156, 0.0044, 0.0092, 0.0095, 0.0078, 0.0083, 0.0042, 0.0039 and 0.0048 respectively.

Effect of different loads on the content of 2-phenylethanol: We found that the content of 2-phenylethanol of 'Vidal' grape showed a downward trend with increasing load during the same period (Fig. 1). On July 1, the highest (0.038 mg/kg) and the lowest content of 2-phenylethanol (0.015 mg/kg) were found in treatments 1 and 5, respectively. On July 15, August 1, August 15, September 1, October 1, and October 15, the highest content of 2-phenylethanol was found in treatment 1, and the corresponding values were 0.042, 0.047; 0.053, 0.057, 0.081, and 0.083 mg/kg, respectively. On September 15, the highest content of 2-phenylethanol (0.069 mg/kg) was found in treatment 4. On November 1, the content of 2-phenylethanol was still decreasing with increasing load, and the corresponding values for treatments 1, 2, 3, 4, and 5 were 0.133, 0.131, 0.129, 0.126, and 0.122 mg/kg, respectively. A significant difference was observed in the content of 2-phenylethanol for different treatments at different times, while the change in the content of 2-phenylethanol in the same treatment showed a rising trend.

Effects of different loads of Vidal on the AADC enzyme content in fruits: It could be seen from (Fig. 2) that before the harvest (October 15), there were two synthesis peaks of the relative expression level of AADC enzyme in the fruit of Vidal with different loads. For treatment 1, the first synthesis peak occurred in the fruit color-changing period (August 1), the second synthesis peak occurred at the beginning of the fruit maturation period (September 15), and the first synthesis peak was lower than the second synthesis peak. For treatment 2, the first synthesis peak occurred in the fruit color-changing period (September 1), the second synthesis peak occurred in the fruit maturation period (October 1), and the first synthesis peak was higher than the second synthesis peak. For treatment 3, the first synthesis peak occurred in the fruit color-changing period (August 1), the second synthesis peak occurred at the beginning of the fruit maturation period (September 15), and the first synthesis peak was lower than the second synthesis peak, which was consistent with the trend of treatment 1. For treatment 4, the first synthesis peak occurred in the fruit color-changing period (September 1), and the second synthesis peak occurred in the fruit maturation period (September 15); the first synthesis peak was lower than the second synthesis peak, and the two synthesis peaks overlapped. For treatment 5, the first synthesis peak occurred in the fruit color-changing period (September 1), the second synthesis peak occurred in the fruit maturation period (October 1), and the first synthesis peak was higher than the second synthesis peak, which was consistent with the trend of treatment 2.

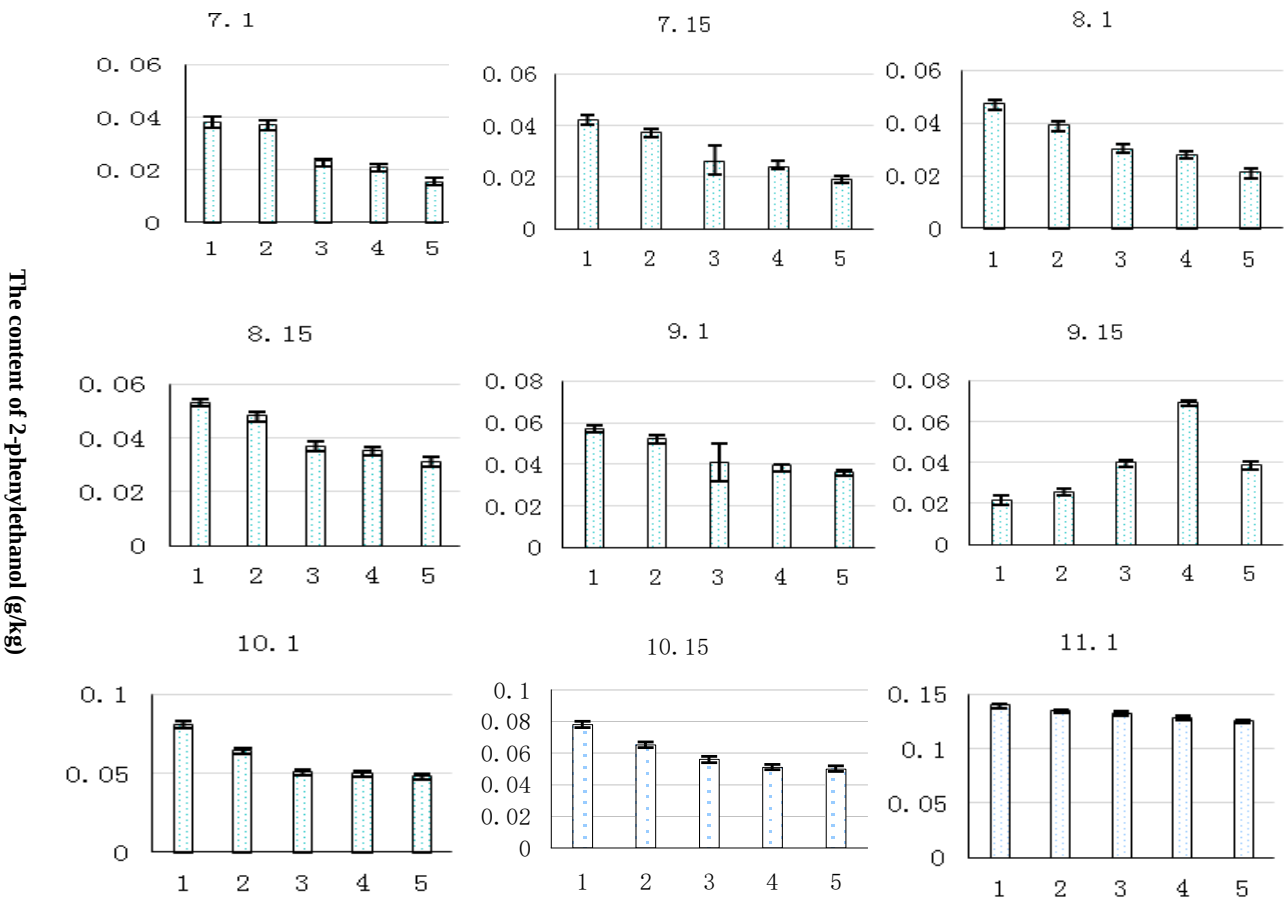

Fig. 1. 2-phenylethanol relative to express different load and growth period on Vidal AADC enzyme relative to express different load and growth period on Vidal.

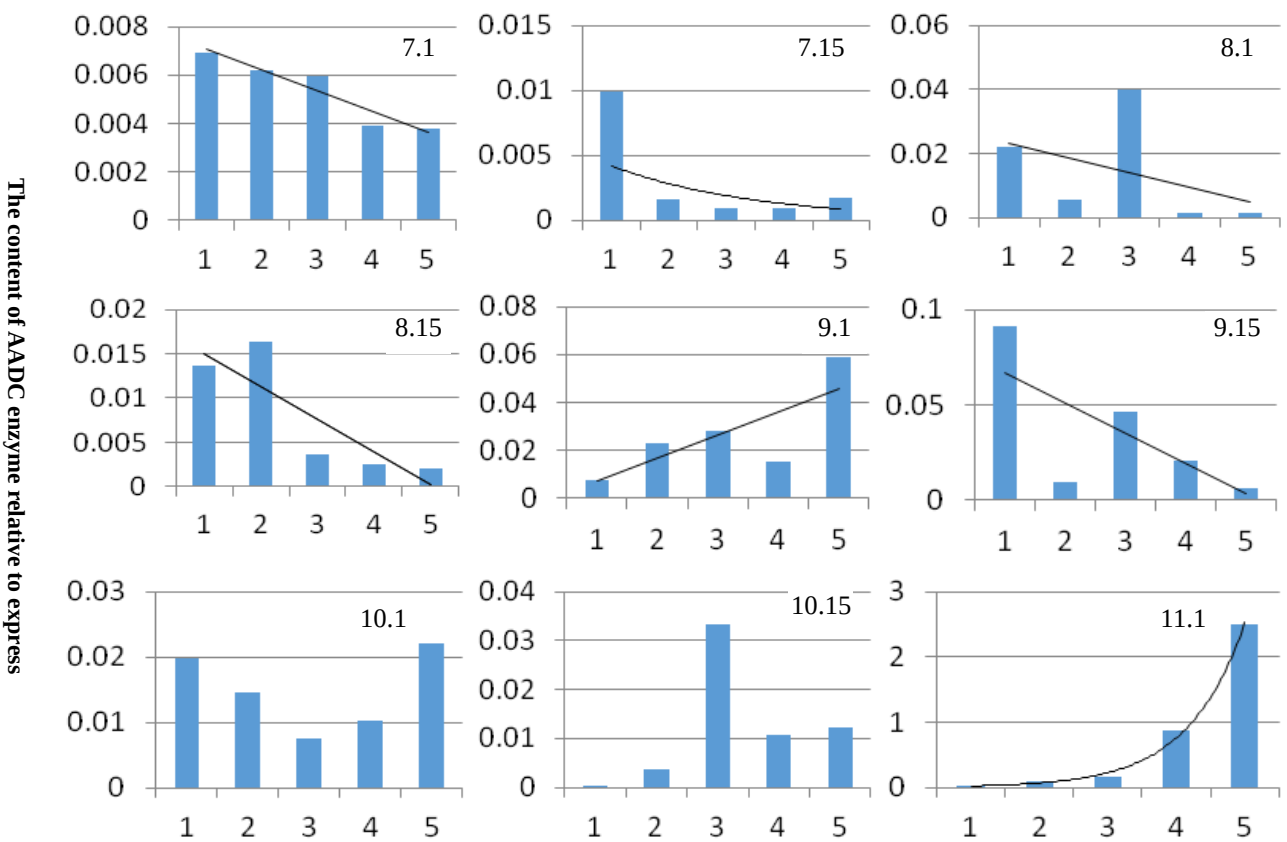

Fig. 2. AADC enzyme relative to express different load and growth period on Vidal.

As shown in Figure 2, significant differences were presented in the relative content of the 2-phenylethanol-regulating enzyme AADC in Vidal with different load treatments at different periods. During the two periods—July 1 and November 1—the relative expression level of enzyme AADC in fruit gradually decreased with increasing fruit load. The trend analysis showed that for different load treatments on July 1, the low fruit load showed a high relative expression level of AADC, whereas the high fruit load showed a low relative expression level of AADC. For different load treatments on November 1, the low fruit load showed a low relative expression level of AADC and the high fruit load showed a high relative expression level of AADC. These results suggested that the load regulation had a direct effect on the relative expression of enzyme AADC. With a small fruit load, the relative expression levels of AADC were high and low in the early and late stages of growth, respectively. With a large load, the content of 2-phenylethanol was low; therefore, the relative expression levels of AADC were low and high in the early and late stages of growth, respectively; the positive regulation was significant in the early stage, and the relative expression level of the enzyme AADC rapidly increased, thus enhancing the positive regulation.

Discussion

Wang *et al*., (2014) identified 27 types of aroma components in the grape juice prepared from the Vidal grapes harvested in maturation period; the dominant types of aroma components were alcohols and ketones, and the relative contents of alcohol and aldehydes were high. Thirty nine types of aroma components were detected in the grape juice prepared from the Vidal harvested in the freezing period, indicating that the aroma components of the grape juice prepared from the fruits harvested in freezing period were more complex and abundant. In the grape juice prepared from the fruits harvested in the freezing period, the most abundant components were esters, followed by alcohols, aldehydes and ketones, and the relative contents of aldehydes and esters were high. Eight types of aroma components were detected in the grape juices of the fruits harvested in maturation and freezing periods, and the content of furfural in the above two kinds of grape juice was relatively high. The results showed that the content of 2-phenylethanol in the grape juices of the maturation and freezing periods was 0 and 0.396, respectively. In this experiment, 2-phenylethanol was detected in the grape juices prepared from the grapes that were harvested in the maturation period and the grapes that were harvested late, and the relative content of 2-phenylethanol was high. The study of Khairallah *et al*., (2016) showed that the delayed harvest allowed the content of 2-phenylethanol in Riesling ice wine to decrease over time, and the changes in the content of 2-phenylethanol in the juice and ice wine over time were not consistent.

The fruit quality of the wine grapes plays a decisive role in the quality of wine. A high fruit load will lead to the premature senescence of grape vines and adversely affect the content of fruit sugar, acid, anthocyanins, and other metabolites, resulting in a decrease in fruit quality (Yuan *et al.*, 2012; Nebauer *et al.*, 2013; Guo *et al.*, 2017). Therefore, it is very important to study the control of optimal loads for different grape varieties. The intrinsic quality of the wine grape fruit is mainly reflected in the content of sugar, acid, and aroma. The results of this study indicated that with increasing load, the content of 2-phenylethanol in the 'Vidal' grape fruits changed irregularly in the dynamic growth period under different load treatments.

Currently, two ADH genes (*Cm-ADH1* and *Cm-ADH2*) and four AAT genes (*Cm-AAT1*, *Cm-AAT2*, *Cm-AAT3*, and *Cm-AAT4*) have been isolated from the cantaloupe melon fruit. All these genes are involved in encoding and expressing the AAT protein in the fruit, and are positively regulated by ethylene (Yahyaoui *et al.*, 2002; Elsharkawy *et al.*, 2005; Manríquez *et al.*, 2006). Real-time PCR analysis indicated that the VvAADC transcript abundance presented a small peak at 110 days after full bloom and then a continuous increase at the berry post-ripening stage, which was consistent with the accumulation of 2-phenylethanol. Hence, we suggest that the AADC-mediated 2-phenylethanol biosynthetic pathway exists in grape berries; however, it possibly contributes minimally to 2-phenylethanol accumulation in post-ripening ‘Vidal blanc’ grapes (Pan *et al.*, 2012).

The effects of different loads on the aroma content of wine grape fruit were as follows:

Owing to the competition between the above-ground and below-ground plant structures for photosynthetic nutrients at the later stage of fruit development in perennial fruit trees, the photosynthetic products are primarily transported to the fruits (storage organ) under high loading conditions. This inhibits absorption of mineral nutrients and moisture, and sufficient nutrients are not available to the root system, which exists in a “hunger” state, and subsequently affects the nutrition levels of the above-ground leaves. Thus, high loading decreases the photosynthesis rate of leaves (Yuan *et al.*, 2012), which reduces the content of 2-phenylethanol and other aromatic components (Lin *et al.*, 2016).

Load, shoot growth, and leaf area showed significant negative correlation. Tree vigor was weakened with increase in loading. Increase in apple load inhibited the growth of new shoots and increased tree canopy volume. This indirectly suggests that excessive loading may inhibit photosynthesis to certain extent (Yuan *et al*., 2012).

AADC levels and activity were found to vary under different loads. During low load loading, AADC levels were high in the early growth phase but decreased towards the later phase. However, accumulated AADC levels under low load treatment were higher than under high load treatment, which resulted in higher levels of 2-phenylethanol and other aromatic substances at low load conditions than in high load conditions.

Conclusions

In summary, the regulation of the load of wine grape ‘Vidal’ in peak fruiting period did not have an impact on 2-phenylethanol content; the dynamic trend of the relative expression level of AADC enzyme during the growth

period of treatment 4 (load: 1250–1500 kg/667 m^2) was consistent with the dynamic content of 2-phenylethanol, and no consistent trends were found for other treatments. In order to ensure fruit quality and related metabolic needs, it is recommended that the suitable load of 'Vidal' grapes for an area of 667 m^2 is 1,000~1,500 kg.

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