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FOLIAR APPLICATION OF AMINO ACIDS MODULATES AROMA COMPONENTS OF 'FUJI' APPLE (*MALUS DOMESTICA* L.)

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Abstract

Volatile flavor compounds play a key role in determining the perception and acceptability as well as enhancing market competitiveness of apple (*Malus domestica* L.). In our study, we evaluated the effects of foliar-applied four different amino acids, i.e. leucine (Leu), isoleucine (Ile), valine (Val) and alanine (Ala), on aroma components and two key enzymes activities involved in aroma metabolism of 'Fuji' apple. The total amount of aromatic components under Ala treatment was significantly higher than those under other treatments. There was a considerable increase in total aroma content, including hexanal, 2-methyl-butanol, nonanal, (E)-2-hexenal, methyleugenol, ethyl acetate, butanoic acid-pentyl ester, butanoic acid-hexyl ester, butyric acid ethyl ester, acetic acid-2-methyl-butyl ester, treated with spraying amino acids compared with the control. More specifically, hexanal, 2-methyl-butanol, methyleugenol and acetic acid-2-methyl-butyl ester exhibited a greater substantial increase of their contents than those of in other ingredients. However, butanoic acid-2-methyl-2-methyl butyl ester maintained a highest level among all aroma components regardless of different amino acids application. Furthermore, the activities of alcohol dehydrogenase (ADH) and alcohol acyltransferase (AAT) were much higher under Ala treatment than those under other treatments. We concluded that foliar-applied organic nitrogen (N), especially for Ala, can improve aroma metabolism and it could be used in production to enhance fruit quality on a commercial scale.

Key words: Aroma component, Alcohol acyltransferase activity, Alcohol dehydrogenase activity, Foliar application, Amino acids, *Malus domestica* L.

Introduction

Aroma is one of the most valuable characteristics and it is considered as an indicator of flavor, maturity and quality of fruit. Aroma comprises of a variety of volatile organic compounds which is vital in mediating the perception of fruit quality and taste. Aroma influences consumers' preference and attracts market-force. Being a crucial trait and representative of fruit quality, strategies to enhance concentrations of aroma have gained considerable ground in recent years (Oritz *et al.,* 2011; Wei *et al.,* 2014; Negri *et al.,* 2015).

Generally, fruit aromatic compounds represent an array of volatile organic compounds among different fruit varieties (Sanz *et al.,* 1996; Schwab *et al.,* 2008). To date, volatile organic molecules components which have been reported are over 300 in apple, 350 in strawberry (*Fragaria* L.), 400 in tomato (*Solanum lycopersicum* L.) and 300 in banana (*Musa nana Lour* L.), respectively (Wick *et al.,* 1969; Mathieu *et al.,* 2009; Aprea *et al.,* 2011; Peano *et al.,* 2014). The most predominant aromatic compounds include esters, aldehydes, lactones, apocarotenoids, alcohols, ketones, terpenoids and sulfur compounds (Pichersky *et al.,* 2006). However, there are several pathways involved in volatile biosynthesis, mainly for metabolism of lipids, amino acids and terpenoids (Venkatesan *et al.,* 2015). Some fruit aroma components, including linear aliphatic alcohols, aldehydes, ketones and esters, are mainly derived from fatty acid oxidation, while other aroma components are synthesized from the amino acid metabolism such as branched chain aliphatic alcohols, ketones, aldehydes and esters (Qin *et al.,* 2014; Shen *et al.,* 2014). It is reported that fruit aroma components are mainly synthesized using amino acids as precursor (valine, leucine, isoleucine, alanine and aspartate) via alcohol acyltransferase (AAT) and aldehyde dehydrogenase (ADH) involved in amino acid metabolism (Echevema *et al.,* 2004; Defilippi *et al.,* 2005). Likewise, different free amino acid concentrations may determine the content of different branched volatiles of fruits (Perez *et al.,* 2002; Gonda *et al.,* 2010; Krogerus *et al.,* 2013; Shen *et al.,* 2014). Some fruit volatile components, for instance, hexanal and 2-hexenal, are reported during early development of fruits, while other esters and alcohols were dominant during fruit ripening (Dixon & Hewett, 2000; Fellman *et al.,* 2000). Changes in AAT activity were positively correlated with total esters contents in fruit during postharvest, especially for acetates and caproate (Wyllie & Fellman, 2000; Perez *et al.,* 2002; Defilippi *et al.,* 2005).

Some exogenously applied inorganic or organic compounds could alter aroma compositions of different fruits (Plaza *et al.,* 2015). For example, exogenously applied nitrogen (N) significantly enhance aromatic expression in *Vitis vinifera L. cv. Sauvignon blanc* wines (Lacroux *et al.,* 2008). Furthermore, addition of N supply caused decreases in contents of 2-phenylethanol and methylbutanol, but increases in ethyl acetate and caproic acid ethyl esters, respectively (Linsenmeier *et al.,* 2005). Similarly, phenylalanine treatment could increase accumulation of benzenoid compounds but decrease contents of C_6 compounds (Garde *et al.,* 2014; 2015; Portu *et al.,* 2015). However, not all exogenous treatment could significantly change the flavor components of plants, for example, Fennel (*Foenculum Vulgare* Mill.) with amma-irradiation treatment (Naeem *et al.,* 2015).

Despite of the immense significance of aroma as an indicator of fruit quality, the physio-biochemical regulation

of this important metabolism urged the exploration of economical and proper management to improve fruit quality. In this study, we reported how and to what extent foliar application of different amino acids could modulate aroma components of 'Fuji' apple fruit. Furthermore, we also reported the changeable pattern of activities of ADH and AAT which are key enzymes involved in aromatic compounds metabolism during fruit ripening.

Material and Methods

Plant materials and trial location: This experiment was conducted in the Experimental Orchard of Northwest A&F University in XunYi County, Shaanxi Province, China. The 'Fuji' apple tree was planted in 2001 and the tree space was 2 m in-row and 3 m between rows. Soil type in this orchard was a loam with organic matter content 14.0 $g \cdot kg^{-1}$, available N content 47.93 $mg \cdot kg^{-1}$, available K content 168.20 $mg \cdot kg^{-1}$, available P content 22.89 $mg \cdot kg^{-1}$ and pH 7.64, respectively (Zhao *et al.,* 2013).

In the present study, 20 trees were randomly selected and divided into five groups of four trees each. Four branches with uniform vegetative growth and fruit load in North-South and East-West directions were selected to conduct foliar application of amino acids from each direction (East, West, North and South). The four amino acids application i.e. leucine (Leu), iso-leucine (Ile), valine (Val) and alanine (Ala) was carried out at four different times (10^{th} May, 10^{th} June, 10^{th} July and 10^{th} August 2014). The selected branches of apple trees were sprayed until drenching and the total volume of each amino acid application was 2500 mL/branch.

Sampling: After two months of foliar treatments (16^{th} October, 2014), fruit samplings were harvested from each branch to investigate aromatic components and enzymes activities. Briefly, eight fruits were randomly sampled from each replicate of each treatment and then were stored at 4°C until analyses.

Determination of AAT activity: AAT activity was measured as described by Fellman (2000) and Pérez (1992). Briefly, 100 mg lyophilized pulp tissue from each sampled fruit was homogenized in 1 mL extraction solution containing 1 mM EDTA, 0.1 M phosphate (pH 8.0), 1% (w/v) PVPP-40 and 0.1% (v/v) Triton X-100. Then the mixture was centrifuged at 25,000×*g* for 15 min at 4 ºC. The supernatant was stored and used for determination of enzyme activities. The reaction mixture for AAT activity assay contained 0.3 mM acetyl-CoA solution, 10 mM butanol solution, 1.6 mM $MgCl_2$ solution and 0.15 mL enzyme extract. The mixture was incubated at 35 ºC for 15 min and then added 100 µL 20 mM DTNB to the reaction system at room temperature for 10 min. Finally, AAT activity was assayed with aid of a spectrophotometer at 412 nm over time. One activity unit (U) was defined as the increase in one unit of absorbance at 412 nm per minute and AAT activity was expressed in U mg $protein^{-1}$ basis.

Determination of ADH activity: ADH activity was determined according to Longhurst (1990). Briefly, 100 mg lyophilized pulp tissue of the fruits were homogenized in 1mL of extraction solution containing 85 mM MES buffer (pH 6.0), 1% (w/v) PVPP-40 and 5 mM DTT. The mixture was centrifuged at 25,000×*g* for 15 min at 4ºC. The supernatant was recovered as enzyme extract. The ADH activity was measured by mixtures with 150 µL acetaldehyde solution (80 mM acetaldehyde in 85 mM MES, pH 6.0), 2.55 mL NADH solution (0.15 mM NADH in 85 mM MES, pH 6.0) and 300 µL enzyme extract. Finally, ADH activity was measured using a spectrophotometer at 340 nm over time. One activity unit (U) was defined as the increase in one unit of absorbance at 340 nm per minute and the ADH activity was defined in U mg $protein^{-1}$ basis.

GC-MS conditions: Aroma constituents were determined using a GC-MS following Cheong *et al.* (2010). 15 mL apple juice, 1 g sodium chloride and 10 µL 3-nonanone (0.04 $mg \cdot mL^{-1}$) were added to 40 mL vials, and then balanced them for 10 min at a constant temperature with 50°C after sealing. Inserted the solid-phase microextraction (SPME) into the sample vials for 2 h. After adsorption at 50°C for 30 min, extracted the SPME and inserted the inlet of gas chromatograph to desorption for 2 min.

The MS apparatus was equipped with fused silica capillary DB-WAX column (diameter 0.25 mm, film thickness 0.25 µm, length 60 m). Column temperature was maintained at 40°C for 2.5 min and then added up to 150°C at the rate of 5°C per min and further increased to 230°C at 10°C per min and kept up at final temperature for 5 min, respectively. Helium (99.99%, 1.0 $mL \cdot min^{-1}$) was the carrier gas. The MS was operated with an EI ion source temperature of 230°C, electron energy at 70 eV, transfer line temperature 230°C and precursor ion 285 $m \cdot z^{-1}$, and activation voltage 1.5 V. The molecular scanning range was 35 to 500 amu. Ionization mode was EI, the ionization voltage 70 eV, ion source temperature 250°C, and the interface temperature was 230°C.

Aroma components were defined by comparing their mass spectra with spectrum of NIST 2011 library. The relative content of each composition was decided by their peak area using 3-nonanone as an internal standard for accurate quantification.

Statistical analysis: One-way analysis of variance for data for each parameter was carried out using the SPSS 20.0 software package. Least significant difference (LSD) test was employed to demonstrate the significant differences at the 0.05 level (95% confidence interval).

Results

Total aroma composition: In this study, 31 kinds of volatile compounds were quantified under control conditions, including 14 esters, 5 alcohols, 4 aldehydes, 4 ketones and 4 other aroma compounds. Esters were the major aroma components of 'Fuji' apple, accounting for 64.55% of total aroma. The types of total aroma components under each treatment were 36, 37, 35 and 34, respectively, while the total contents of aroma substances under various process conditions were increased by 3.97, 4.52, 5.53 and 9.18%, respectively ($p<0.05$). Nonetheless, the total aroma components exhibited peak values with Ala treatment while the lowest values were found in response to Leu treatment (Fig. 1).

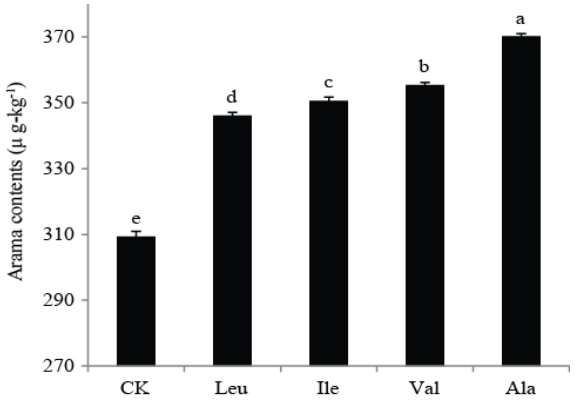

Fig. 1. Effects of foliar-applied different amino acids, i.e. leucine (Leu), isoleucine (Ile), valine (Val) and alanine (Ala), on aroma components of 'Fuji' apple fruit. Means followed by a different letter are significant difference by Duncan's Multiple Range Test (p<0.05); n = 3, mean ± standard deviation.

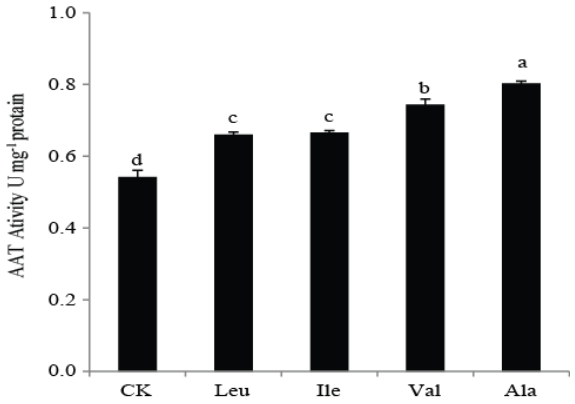

Fig. 2. Effects of foliar-applied different amino acids, i.e. leucine (Leu), isoleucine (Ile), valine (Val) and alanine (Ala), on alcohol acyltransferase (AAT) activity of 'Fuji' apple. Means followed by a different letter are significant difference by the Duncan's Multiple Range Test (p<0.05); n = 3, mean ± standard deviation.

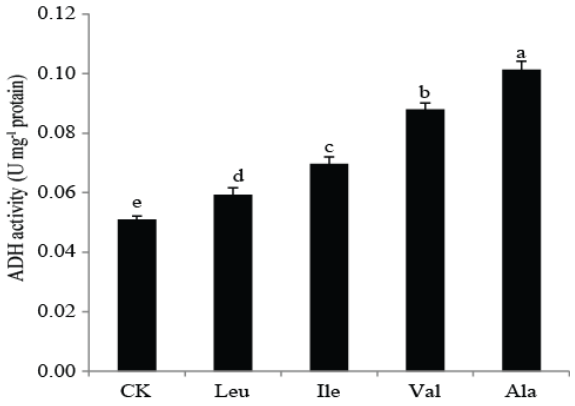

Fig. 3. Effects of foliar-applied different amino acids, i.e. leucine (Leu), isoleucine (Ile), valine (Val) and alanine (Ala), on fruit alcohol dehydrogenase (ADH) activity of 'Fuji' apple fruit. Means followed by a different letter are significant difference by the Duncan's Multiple Range Test (p<0.05); n = 3, mean ± standard deviation.

Esters: Some aroma components, especially esters, play a significant role in determining flavor characteristics of fruit. The ester compounds exhibited a substantial improvement after amino acid spraying, such as hexanoic acid-propyl ester, butanoic acid-2-methyl-2-methyl butyl ester and acetic acid-2-methyl-butyl ester. In contrast, the compounds that exhibited considerable decrease were acetic acid, 2-methyl butyl ester, butanoic acid-2-methyl-butyl ester, respectively. A significant increase in ester content was observed only under Ala treatment as compared to other treatments (Table 1).

Alcohols: An increase in total content of alcohols was recorded under different amino acid treatments, especially for Val and Ala treatments. In addition, the content of 2-methyl-butanol exhibited a greatest increase among four amino acids application treatments (Table 1).

Aldehydes: The evident changes were recorded in aldehyde accumulation after foliar application of all amino acids. Aldehyde contents were increased under both Ile and Ala treatments. The volatile compounds, hexanal, (E)-2-hexenal, showed significant increase after four amino acid spraying. The content of nonanal was delectable and showed minute fluctuation only in both Ile and Ile treatments. Most treatments caused accumulation of butanal except for Ala treatment (Table 1).

Other components: In addition to the above mentioned, other fragrance ingredients, including ketones, ethylbenzene, 1,3-dimethylaniline, farnesene, anethole and methyleugenol, were increased in response to application of all amino acids. Some volatile compounds, such as farnesene and methyleugenol, were increased, while acetic acid, 2-methyl butyl ester, butanoic acid-2-methyl-butyl ester decreased after four amino acid application (Table 1).

AAT and ADH activities: As shown in Fig. 2 and Fig. 3, as compared with the control, all treatments imposed a significant impact on AAT activity in fruit. The maximal values of AAT and ADH activities were both found under Ala treatment (Figs. 2, 3).

Discussion

More than 350 types of aroma substances have been detected in apple fruit, most importantly for acetic acid-2-methyl butyl ester, butanoic acid-2-methyl-ethyl ester, 2-hexenal, butanoic acid-2-methyl-butyl ester, 3-nonanone and 2-caproaldehyde. These aroma components are essential indicators of apple quality (Nie *et al.,* 2006; Li *et al.,* 2008).

Each species has a characteristic aroma component at ripening stage (Li *et al.,* 2008). Low molecular weight esters are the major aroma components of apple fruit, which account for 78-92% of total volatile substances (Ferreira *et al.,* 2009; Farneti *et al.,* 2015). Hexanal was one of the main volatile substances of apple fruit before ripening, while esters and alcohols gradually became the dominant components at/after fruit ripening. An increase in volatile components during fruit ripening could be an indicator of fruit maturity (Aguiar *et al.,* 2014; Yilmaztekin *et al.,* 2015). Moreover, esters are principal volatile substances that contribute maximally to form peculiar and specific aromas of 'Fuji' apple, which were called 'Ester

aroma type' represented with most low-molecular weight esters (Du *et al.,* 2015). Certain amino acids, i.e. isoleucine, leucine and valine, are reported to be involved in branched-chain synthesis (Ei Hadi *et al.,* 2013). For example, the most important substances of yeast strain (*S. pastorianus*) are valine, isoleucine, cysteine, glutamine, leucine and proline (Procopio *et al.,* 2013). In banana, the accumulation of Val and Leu showed a significant increase by 2-3 folds during ripening. Leucine is reported to be converted into the 3-methyl-1-butanol, 3-methyl-butyl and other substances, valine is converted into 2-methyl-propionic acid and other substances when volatiles of banana were synthesized *In vitro* (Tressl & Drawert, 1973). Some composition contents, including isoamyl alcohol, isoamyl acetate and 2-methylbutyl acetate, were increased significantly with leucine application, while the application of valine and isoleucine increased the production of isobutanol and 2-methyl butanol, respectively (Procopio *et al.,* 2015). The content of 2-methyl butyl ester, the main aroma component, was increased by 2-folds when isoleucine was applied (Rowan *et al.,* 1996). The content of aroma compounds was influenced by different N levels and varieties of melon (Xu *et al.,* 2009; Qian *et al.,* 2011). In our study, the highest total content of aroma components was recorded with Val spray. In agreement with this, the production of aroma-active compounds in yeast was found to be affected by supplying amino acid during lager yeast fermentation (Procopio *et al.,* 2015).

Table 1. Changes in aroma composition of 'Fuji' apple in response to foliar applied different amino acids.

Aroma composition	Foliar treatments				
($\mu g \cdot kg^{-1}$)	Control	Leu	Ile	Val	Ala
Ester					
Methyl hexadecanoate	5.11±0.01c	5.01±0.04c	4.04±0.06d	5.98±0.08b	0.97±0.08a
Ethyl acetate	ND	ND	0.21±0.01	ND	0.34±0.01
Acetic acid, 2-methyl butyl ester	44.93±0.35a	4.56±0.12bc	2.73±0.15d	4.05±0.05c	4.77±0.19b
Butanoic acid, 2-methyl-, hexyl ester	8.61±0.35b	8.06±0.04c	8.29±0.10bc	9.04±0.05a	7.99±0.04c
Propyl 2-propenoate	0.22±0.01b	0.21±0.01b	ND	0.38±0.02a	ND
Acetic acid, 5-hexen- l ester	0.45±0.01b	0.82±0.02a	0.45±0.01b	ND	0.83±0.01a
Acetic acid, butyl ester	2.61±0.03a	1.58±0.04c	1.16±0.04d	1.61±0.08c	1.88±0.01b
Butanoic acid, propyl ester	1.81±0.01c	1.85±0.03b	1.59±0.03c	1.13±0.06d	2.33±0.04a
Butanoic acid, pentyl ester	0.71±0.01a	0.67±0.03a	ND	0.71±0.03a	0.13±0.02b
Butanoic acid, butyl ester	ND	0.57±0.03d	0.64±0.02c	0.91±0.04b	1.02±0.02a
Butanoic acid, 2-methyl-, butyl ester	1.87±0.01d	2.9±0.09ab	3.17±0.12a	2.22±0.13c	2.81±0.08b
Hexanoic acid, propyl ester	ND	32.85±0.33c	39.69±0.24b	43.11±0.82a	41.22±1.29ab
Butanoic acid, hexyl ester	2.38±0.02b	2.76±0.22ab	2.88±0.13a	1.49±0.09c	0.85±0.05d
Butanoic acid, 2-methyl- ethyl ester,	ND	17.68±0.49c	20.51±0.46b	19.88±0.37b	21.7±0.29a
Butanoic acid, 2-methyl-, 2-methyl butyl ester	34.84±0.53d	84.4±0.63a	74.59±0.77c	78.32±0.58b	85.82±0.50a
Butyric acid ethyl ester	ND	ND	0.15±0.02b	0.41±0.01a	ND
Butanoic acid, 2-methyl-, ethyl ester	85.95±0.19a	24.73±0.41b	22.83±0.49bc	22.04±1.27c	22.51±0.34c
Acetic acid, 2-methyl-, butyl ester	ND	19.38±0.42b	20.03±0.06b	21.42±0.60	19.64±0.40b
Hexanoic acid, 3-methyl-, butyl ester	3.79±0.01c	3.87±0.11bc	4.03±0.05ab	4.07±0.05ab	4.16±0.09a
Alcohol					
Ethanol	0.24±0.03c	0.45±0.03b	ND	0.29±0.01c	0.54±0.01a
Isobutyl alcohol	8.53±0.01c	10.70±0.30a	9.87±0.15b	9.30±0.20b	7.18±0.13d
Butanol, 2-methyl-	ND	22.72±0.24c	26.94±0.08b	33.17±0.46a	32.69±0.76a
1-Hexyl alcohol	8.39±0.32bc	8.84±0.22bc	7.31±0.16c	11.31±0.97a	9.46±0.44b
1-Butanol	1.34±0.14a	0.82±0.05c	0.56±0.04d	1.09±0.01b	1.32±0.01a
trans-2-hexen-1-ol	ND	0.04±0.00	0.05±0.01	ND	ND
Aldehyde					
Hexanal	ND	6.17±0.16c	5.77±0.19c	6.75±0.15b	7.75±0.14a
Butanal	0.48±0.08c	0.12±0.02d	0.69±0.02b	1.15±0.05a	ND
2-Caproaldehyde	39.35±0.31a	30.74±0.25c	36.23±0.61b	34.94±0.30b	39.02±1.47a
Hexen-2-al	34.31±1.08a	34.73±0.83a	35.81±0.81a	29.49±0.59b	33.69±0.96a
Nonanal	ND	ND	0.08±0.09	ND	ND
(E)-2-Hexenal	2.66±0.03a	1.15±0.05c	0.30±0.05d	1.36±0.04b	1.33±0.03b
(E)-2-Octenal	ND	2.05±0.04b	2.65±0.04a	1.44±0.05c	1.96±0.09b
Ketone					
3-Nonanone	10.21±0.31a	11.04±0.16a	11.79±0.84a	12.07±0.63a	10.37±0.61a
Damascenone	0.07±0.00c	ND	0.04±0.00b	0.73±0.004a	ND
trans-geranyl acetone	0.69±0.03a	ND	0.69±0.01a	ND	0.31±0.01b
E-á-beta-ionone	0.11±0.03a	0.12±0.01b	ND	0.11±0.01b	ND
Other composition					
Ethylbenzene	0.10±0.01a	0.06±0.01b	0.11±0.01a	ND	0.03±0.01b
1,3-dimethylaniline	2.16±0.16a	1.73±0.09b	2.07±0.06a	1.05±0.04c	1.75±0.06b
Anethole	0.46±0.02a	0.36±0.01b	0.45±0.02a	ND	ND
Methyleugenol	ND	11.14±0.08a	10.82±0.77a	8.79±0.18b	11.56±0.75a
Farnesene	6.99±0.08c	7.38±0.33bc	8.90±0.14a	7.76±0.15b	7.95±0.06b

All the results were expressed as means ± standard deviation of three replications. Values in a column followed by different letters show significant difference based on Duncan's Multiple Range test at 0.05 level. Following abbreviations were used for different amino acids treatments: leucine (Leu), isoleucine (Ile), valine (Val), alanine (Ala)
ND: Not detected

Most flavor and ester characteristics of aroma components are believed to be generated by amino acid metabolism, which require two-step enzyme reaction involving ATT and ADH (Sanz *et al.,* 1997; Ei Hadi *et al.,* 2013). The AAT activity played a critical role in amino acids metabolism to synthesize fruit aroma. Alcohol dehydrogenase (ADH) is an important enzyme of lipoxygenase (LOX) pathway, which can convert aldehydes into alcohols through alcohol dehydrogenation and then synthesize esters as precursors in plants (Venkatesan *et al.,* 2015). Our results showed low activities of ADH during apple maturity across all treatments. The rate of ethanol production was associated with ADH activity at only low level, while it is unrelated to higher ADH activity (Defilippi *et al.,* 2005). This may be sufficient to synthesize alcohols when ADH activity reached to a certain level. We concluded that the great increase of ester content was cause by increase of substrate concentration rather than enzyme activity. In an early study, the selective properties of ADH and AAT could control the reduction and formation step of ester involved in aroma substances synthesis in banana (Wyllie *et al.,* 1996). Overall, AAT and ADH activities with Ala treatments were much higher than those in the other treatments.

Conclusion

Fruit aroma is an important indicator to reflect fruit quality and flavor. A total of 31 volatiles were determined of 'Fuji' apple fruit in our study. The species and contents of aroma components after spraying amino acids exhibited a significant increase, especially for butanoic acid-2-methyl-2-methyl butyl ester regardless of different amino acids treatments. Both AAT and ADH activities were increased when treated with Ala. Therefore, the foliar application of Ala could be used as a commercial practice to improve aroma metabolism in apple.

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