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Biosynthesis of Strawberry Aroma Compounds through Amino Acid Metabolism

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The fate of amino acids in relation to aroma biogenesis was studied in strawberries using the in vitro growth approach. This fruit presented differences in the level of metabolization for different amino acids. Incubations of strawberries with L-isoleucine gave rise to an increase of fourteen compounds in this fruit aroma, either not detected previously or constituents of strawberry aroma. However, L-valine incubations did not provide a significant change in this fruit aroma. Strawberry feeding with L-isoleucine resulted in a 7-fold increase in the sum of 2-methylbutanoate esters, and a double production of 2-methylbutyl esters compared to those of control fruits. Around 94% of the ester increase corresponded to 2-methylbutanoates, with ethyl 2-methylbutanoate being the most representative compound (92%). On the other hand, among the 2-methylbutyl esters, comprising around 6% of total aroma volatiles increase, 2-methylbutyl acetate was the major compound (95%) arising from L-isoleucine strawberry feeding. The role of enzymatic activities within the amino acid metabolic pathway in strawberry fruits is discussed.

KEYWORDS: Strawberry; ester; biosynthesis; amino acid

INTRODUCTION

The aroma of cultivated strawberries is an important attribute that greatly influences consumer acceptability. Volatile esters constitute the largest group of compounds identified in strawberry aroma. They are formed by esterification of alcohols and carboxylic acids catalyzed by the enzyme alcohol acyltransferase (AAT)(*1*−*3*). One of the main sources of substrates for this ester biosynthesis is the metabolism of amino acids, generating alcohols, aldehydes, and acids, either aliphatic, branched, or aromatic. These compounds contribute, and in some cases are determining, to the primary aroma of many fruits. Free amino acid content changes have been demonstrated to occur during ripening, which is when characteristic aroma is produced in most fruits. Thus, the free amino acids pool could account for the different aroma profile patterns found in fruits. We have previously observed in Chandler strawberries a 4-fold increase in ethyl esters concomitant to a 10-fold decrease in alanine content during ripening (*4*). Alanine was the major free amino acid found in this fruit jointly to asparagine and glutamine, acting as nitrogen reservoirs and sources in the cells. Metabolism of amino acids toward aroma biogeneration seems to occur via two consecutive enzymatic steps: deamination and decarboxylation. Tressl and Drawert's (*5*) studies on banana showed that labeled leucine is converted into labeled 3-methylbutanol, 3-methylbutanoic acid, and 3-methylbutyl esters. A first step of transamination is inferred from the experimental data as glutamic acid is produced from 2-oxoglutarate. This hypothesis was validated after identification of the corresponding intermediary 2-oxoacid from the amino acid and the isolation of alanine 2-oxoglutarate aminotransferase in tomato (*6*). This mechanism of action seems to be in good agreement with those elucidated for other plant and animal aminotransferases (*7*).

The next step is the decarboxylation of the 2-oxoacid formed after amino acid transamination. Drawert (*8*) postulated that the 2-oxoacid oxidative decarboxylation occurs by an enzymatic complex similar to pyruvate dehydrogenase or 2-oxoglutarate dehydrogenase from the TCA cycle, involving as cofactors thiamin pyrophosphate (ThPP), lipoic acid, FAD, NAD, and coenzyme A. This complex would produce 3-methyl-butanoyl-CoA from leucine, a substrate for alcohol acyltransferase to 3-methyl-butanoate esters. But the actuation of an enzyme similar to pyruvate decarboxylase, from the fermentative pathway, producing the non-oxidative decarboxylation of 2-oxo-acids to form aldehydes is possible as well. In this sense, Yoshioka et al. (*9*) proposed a 2-oxo-isocaproate decarboxylase, having ThPP as cofactor, as being responsible for 3-methyl-butanal synthesis in banana. This aldehyde is quickly reduced to the corresponding alcohol by NADH-alcohol dehydrogenase, and it was the labeled moiety found in banana esters.

Strawberry is classified as a nonclimateric fruit on the basis of a lack of increased respiration and ethylene production as the fruit changes color, texture, and flavor (*10*). Direct study of nonclimateric fruits such as strawberries has been limited because ripening generally does not continue normally following detachment. In this sense, Perkins-Veazie and Huber (*11*) provided an in vitro system to study nonclimateric fruit

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development that allows incorporation of substances to study fruit ripening (*12*−*14*). Based on this in vitro approach, we have previously studied the biosynthesis of furaneol in strawberry through the incubation of whole fruits with different precursors (*15*). The importance of fruit age for in vitro culture, both for culture medium uptake by the fruit and for precursor metabolization, has been observed in previous studies. The aim of the present work was first to establish the most suitable fruit age for performing studies on precursors and metabolic intermediates for ester biosynthesis in strawberries, and second, to determine the physiological fate of amino acids in whole intact strawberries in terms of aroma biosynthesis.

EXPERIMENTAL PROCEDURES

Fruits. Strawberry fruits (*Fragaria* × *ananassa* Duch.) cultivar Camarosa, were grown in Torreagro fields (S. Bartolomé de la Torre, Huelva, Spain), and flowers were marked at blooming. Fruit age was expressed as days after blooming (dab).

In vitro Growth. The in vitro experiments were carried out using the system developed by Perkins-Veazie and Huber (*11*). Fruit peduncles were trimmed to a uniform length (ca. 40 mm) and immersed in a vial containing growth medium (88 mM sucrose). In amino acid metabolism studies, this growth solution was supplemented with L-valine or L-isoleucine at a concentration of 0.1 M. Fruits were incubated for 48 h and then transferred to vials with growth medium for five more days. Strawberry fruits were grown in a growth chamber at 22−23 °C, 16 h photoperiod, 300 $\mu E \cdot m^{-2} \cdot s^{-1}$ light, and 85% relative humidity.

Sugars. Main sugars from strawberries were studied. Two ethanolic extracts of quarters from 5 strawberries were obtained per sampling day. Ethanolic extracts, two determinations each, were fractionated and analyzed by HPLC according to Pérez et al. (*16*).

Analysis of Volatile Compounds. Five strawberries were placed in a hermetically closed container (600 mL) housed within a thermostated water bath (25 °C). After 10 min equilibrium time, volatile compounds were adsorbed on a SPME fiber (100-μm poly(dimethylsiloxane)). Sampling time was 20 min, and sampling was carried out in duplicate. Desorption of volatile compounds trapped in the SPME fiber was done directly into the GC injector. Volatiles were analyzed using a HP-5890 gas chromatograph equipped with a fused silica capillary column DB-Wax (30 m × 0.25 mm). Quantitation was performed using individual calibration curves for each identified compound. Compound identification was carried out on a GC−MS Fison series 8000 equipped with a similar stationary phase column and two different lengths, 30 and 60 m, matching against the Wiley/NBS Library and by GC retention time against standards.

Extraction of Alcohol Acyltranferase (AAT) Activity and Assay. Preparation of enzyme crude extracts and AAT activity spectrophotometric assays were carried out according to Pérez et al. (*2*) in duplicate. Strawberries were cut symmetrically into four pieces. Five pieces from five different fruits were mixed in a Waring blender with polyvinyl polypyrrolidone and 0.5 M Tris−HCl pH 8.0 buffer containing 0.1% Triton X-100. The resulting homogenate was vacuum-filtered through a Whatman No. 1 filter paper, and the residue was washed twice with the above-described buffer. The extract was centrifuged at 27 000*g* for 20 min, and the supernatant was used as the crude extract.

The standard assay mixture for the assay of AAT activity consisted of 0.85 mL of 0.5 M Tris−HCl pH 8.0 buffer containing 11.6 mM $MgCl_2$, 0.3 mM acetyl-CoA, 10 mM butanol, and 0.15 mL of the enzyme solution. The mixture was incubated at 35 °C for 15 min, and then 50 μL of 20 mM 5,5′-dithiobis(nitrobenzoic acid) was added, and the mixture was allowed to stand at room temperature for 10 min. The increase in absorbance at 412 nm over time was measured.

RESULTS AND DISCUSSION

The in vitro culture approach has demonstrated to be quite useful for the study of the biosynthetic pathway producing furaneol and derivatives in strawberry, which are a very important class of compounds for this fruit aroma (*15, 17, 18*). This technique was assessed as a possibility to examine biochemical pathways involved in the biogenesis of aroma esters in strawberry. Strawberry fruits of different ages (dab) were grown in vitro, and their ability to synthesize esters at different days of in vitro culture was assessed (**Figure 1**). Ester production by in vitro grown fruits was compared to that of fruits attached to the plant (in vivo) and having similar age. After 5 days of in vitro culture, fruits started to present signs of deterioration, mainly due to desiccation. These signs of deterioration began earlier as fruit used for in vitro culture was older. Thus, strawberry in vitro culture was carried out for no longer than a week. Fruits with 19 and 22 dab did not show any significant increase in ester production during culture for 7 days. Similarly, 29 dab fruits showed almost no change in ester production after 2 days of in vitro culture. On the other hand, ester production increased during in vitro culture when fruits with 24 and 26 dab were used. This fruit age seemed to be optimum for in vitro studies, as in both cases an ester production increase was observed coinciding with a similar increase in fruits on the plant (in vivo). However, as shown in **Figure 2**, fruits older than 24 dab showed a decrease in fruit weight during in vitro culture, so that it is not clear whether culture medium was imbibed in the fruit. In contrast, 24 dab strawberries increased fruit weight after 2 and 5 days of in vitro culture, decreasing slightly thereafter as the fruit deteriorated. Considering the high rate of transpiration that strawberry fruits present and the increase in fruit weight observed for 24 dab fruits during the first 5 days of in vitro culture, it seems clear that culture medium was imbibed in the fruits during the in vitro culture. Actually, it was observed that an average daily uptake of 135 μL of culture medium occurred per gram of fruit weight.

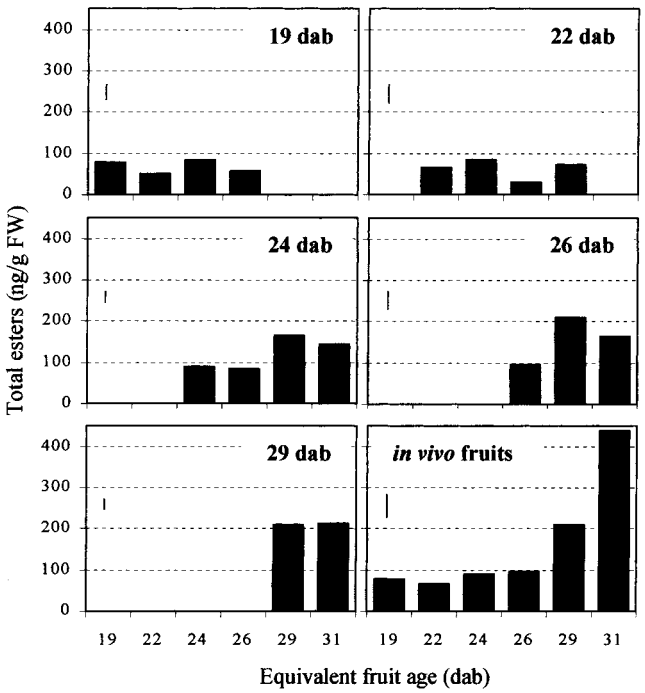

Figure 1. Total esters production of strawberries of different ages (19–29 dab) during in vitro growth for up to 7 days. Comparison with ester production by strawberries grown on the plant (in vivo fruits) with equivalent fruit age. Vertical bars represent least significant differences at $p < 0.05$.

The activity levels of the enzyme responsible for ester production, AAT, were also determined in in vitro grown 24 dab strawberries. Fruits showed an increase in AAT activity during in vitro strawberry growth, although not to the same

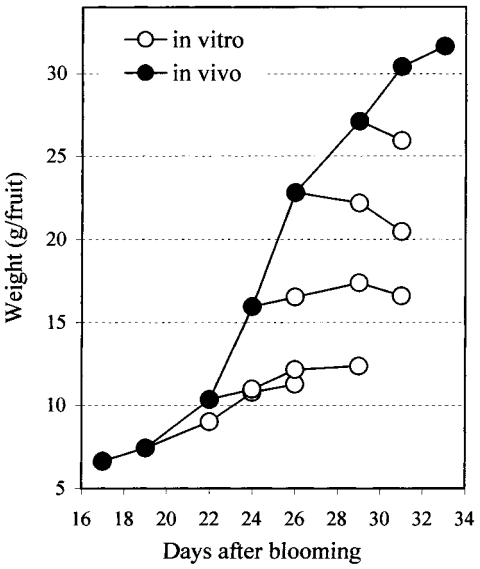

Figure 2. Weight of strawberries grown on the plant (in vivo, ○ open circles) and in vitro (● solid circles) for up to 7 days.

extent as that in in vivo fruits. After 5 days of in vitro culture, AAT activity was 4.47 mU/g FW, much lower than the level found for the corresponding in vivo age (fruits at 29 dab, 20.19 mU/g FW). The level of AAT increased to 23.35 mU/g FW after 7 days of in vitro culture, almost half of the AAT activity found in 31 dab fruits (44.03 mU/g FW). However, as shown in **Figure 1**, ester production in in vitro grown fruits did not increase accordingly to AAT levels as it seemed to occur in in vivo fruits. Ester production was 163.5 ng/g FW at day 5 of in vitro culture, relatively close to the production of esters in fruits on the plant for 29 dab (211.1 ng/g FW). At day 7 of culture, in vitro grown strawberries produced 142.6 ng esters/g FW, almost a third of the production by 31 dab fruits in vivo. According to these data, substrate concentration in the fruits seems to be a limiting factor for ester production at least in in vitro grown strawberries. This limitation might be for substrates of AAT, that is alcohols and/or acyl-CoAs, or precursors for these classes of compounds.

As sink organs, fruits are dependent on the translocation of sucrose, amino acids, and organic acids to their cells. Developing strawberry fruit depends on the delivery of photoassimilates from the leaves. The rate of import of these photoassimilates from the leaves is governed by the metabolic activity of the fruit (*19*) as the photosynthetic carbon dioxide fixation in the fruit chloroplasts would make only a minor contribution to fruit growth as observed in tomato fruit (*20*). Thus, the lack of amino acids in the culture medium used in the preliminary experiments could account for the indirectly deduced limitation of substrates for ester biosynthesis.

Sucrose is the major sugar translocated in strawberry and may be unloaded into the apoplast (*21, 22*). Despite using sucrose as the carbon source in the culture medium, this sugar did not accumulate in the fruit, as shown in **Figure 3**. Conversely, glucose and fructose levels determined for in vitro grown strawberries were quite similar to those found in fruits with the same relative age (in vivo fruits). In this sense, the enzyme acid invertase has frequently been implicated in apoplastic phloem unloading, so that sucrose is converted to hexoses, thereby allowing the continued exit of sucrose from the phloem (*23*). Differences found among sugars contents within in vitro grown

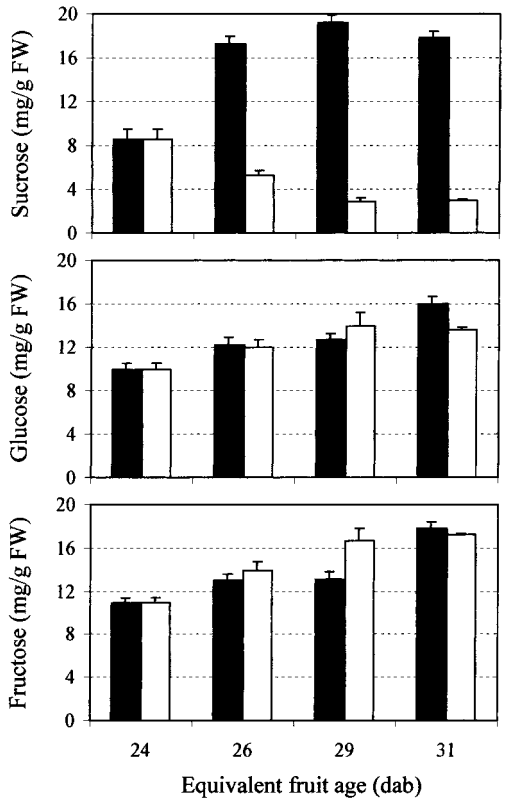

Figure 3. Contents of the main strawberry sugars during in vitro growth (solid bars) compared to fruits on the plant (in vivo) of the same relative age (open bars).

fruits might suggest a very strong regulation of this enzyme to provide physiological levels of both glucose and fructose in the fruit cells.

On the basis of the increase in ester production, AAT activity, and medium uptake results, 24 dab strawberries were selected as the most suitable fruits to be used for strawberry in vitro culture in order to perform studies on the metabolic fate of amino acids in this fruit. For this purpose, strawberries with this age were grown in vitro as stated in Experimental Procedures and the culture media were supplemented with 0.1 M L-isoleucine or 0.1 M L-valine. These two amino acids were selected because they have been shown to be aroma precursors in fruits such as banana and tomato, giving rise to branched esters. Strawberry aroma is quite poor in volatile esters derived from these amino acids skeletons. The other two possible ester biogenetic amino acids, L-leucine and L-alanine, were not selected because relatively higher contents of compounds derived from the former (e.g., 3-methylbutyl acetate and butanoate) can be found in strawberry aroma, and L-alanine might be a source of ethyl esters, which are very abundant in this fruit aroma.

Aroma volatiles, either constituents or metabolically formed after incubation with L-isoleucine and L-valine, were identified and quantified. **Figure 4** shows representative HRGC chromatograms of the fruits' headspace after 5 days of in vitro culture. No quantitative or qualitative differences were found in the aroma of the control and the L-valine fed fruits. However, feeding strawberries with 0.1 M L-isoleucine produced an increase in the expected metabolites derived from this amino acid (**Figure 4** and **Table 1**). Incubation with this amino acid made fruits actively emit 2-methylbutanal and 2-methylbutanol for the first 48 h (data not shown). Levels of these compounds decreased steadily thereafter as esters derived from these

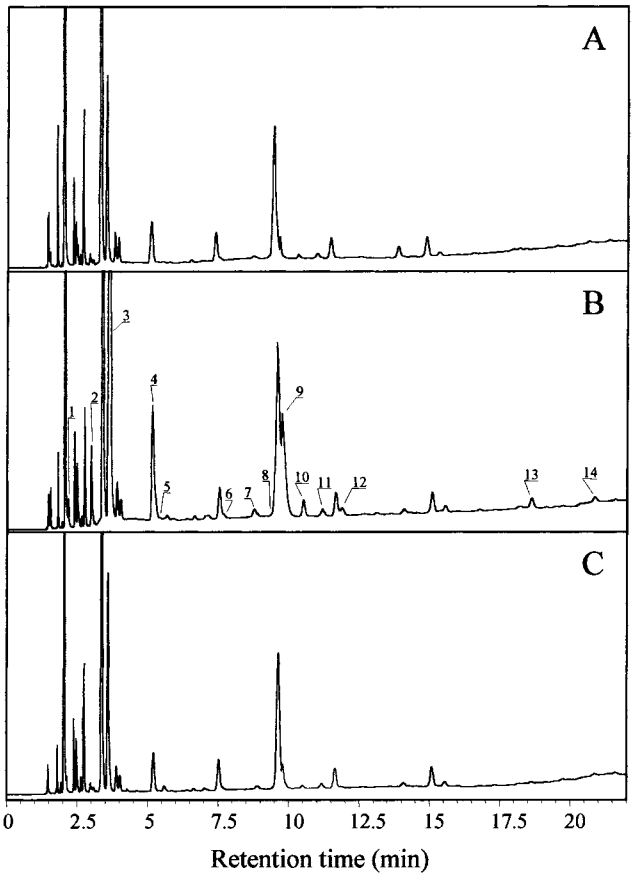

Figure 4. Typical GC–FID aroma profiles of strawberries grown in vitro for 5 days in media containing (A) 88 mM sucrose; (B) 88 mM sucrose and 0.1 M L-isoleucine for the first 48 h; (C) 88 mM sucrose and 0.1 M L-valine for the first 48 h. Peak numbers in chromatogram B correspond to biosynthetic compounds in **Table 1**.

Table 1. Changes in the Concentrations of Aroma Compounds (pmol/g FW) from L-isoleucine Fed Strawberries

peak	biosynthetic compound	control[a]	L-isoleucine
2	methyl 2-methylbutanoate	69.70± 5.58	535.70± 21.43
3	ethyl 2-methylbutanoate	930.96 ± 37.24	6749.96 ± 249.75
5	propyl 2-methylbutanoate	1.32± 0.33	33.32± 5.66
8	butyl 2-methylbutanoate	tr	2.56± 0.64
12	2-methylbutyl 2-methylbutanoate	0.82± 0.25	9.52± 1.52
13	(*Z*3)-hexenyl 2-methylbutanoate	2.58± 0.67	16.08± 1.61
	total 2-methylbutanoate esters	1005.39	7347.14
4	2-methylbutyl acetate	272.86± 13.64	638.86± 24.92
6	2-methylbutyl propanoate	5.08± 1.06	9.08 ± 1.36
11	2-methylbutyl butanoate	8.39± 1.51	10.59 ± 1.53
14	2-methylbutyl hexanoate	2.48± 0.68	8.48 ± 1.52
	total 2-methylbutyl esters[b]	289.61	676.51
1	2-methylbutanal	nd	269.12± 3.46
7	2-methylbutanol	nd	87.11± 6.09
9	ethyl 2-methyl-2-butenoate	14.71± 2.35	71.51± 5.72
10	2-methyl-2-butenyl acetate	5.43± 1.09	24.23± 4.12

[a] nd, not detected; tr, traces. [b] Including 2-methylbutyl 2-methylbutanoate.

compounds started to appear increasingly for 5 days of in vitro culture. Thus, specificity for amino acid metabolization in strawberry is inferred, either at the aminotransferase or decarboxylating steps, as no 2-methylpropanal (the expected compound resulting after consecutive deamination and decarboxylation of L-valine), nor derived esters, were detected. **Table 1** shows that strawberry feeding with L-isoleucine resulted in more than a 7-fold increase in the sum of 2-methylbutanoate esters, and more than double the production of 2-methylbutyl esters. Apparently, metabolization of L-isoleucine by strawberry is mainly carried out through the decarboxylating branch producing acyl-CoAs after the deamination step. Around 94% of the ester increase corresponded to 2-methylbutanoates arising from this branch, with ethyl 2-methylbutanoate being the most representative compound (92%). On the other hand, 2-methylbutyl esters, representing close to 6% of the total compounds arising from L-isoleucine strawberry feeding, would be theoretically produced through the decarboxylating branch producing aldehydes. These esters were mainly represented by 2-methylbutyl acetate (95%). Another explanation for these differences found in the nature of esters arisen from L-isoleucine could be the kinetic characteristics of AAT. We have found K_m values for acyl-CoAs an order of magnitude lower than those for alcohols of the same chain length.

These data on ester distribution are not in agreement with the observations made by Drawert and Berger (*24*), who found that 2-oxopentanoic acid was a powerful alkylating agent in strawberry segments after decarboxylation. Thus, the decarboxylating branch producing aldehydes would be prevalent in strawberry. However, it is also possible that added 2-oxopentanoic acid was reduced by other oxido-reductases before entering the ester synthesis pathway. Similar discrepancies can be found in ester metabolic studies on apple. Rowan et al. (*25*) observed that incubation of Granny Smith apple with L-isoleucine or 2-methylbutanoic acid produced almost exclusively ethyl 2-methylbutanoate (98% and 84%, respectively). However, Red Delicious apple produced predominantly 2-methylbutyl esters in the same conditions. These authors attributed this predominance of 2-methylbutyl esters to a consequence of the irreversible reduction of 2-methylbutanoic acid to 2-methylbutanol occurring in this variety. However, Berger and Drawert (*26*) found that incubations with 2-oxopentanoic acid of Red Delicious apple disks produced more butanoate esters than butyl esters through decarboxylation, but with a different conversion rate of 67% and 21%, respectively.

Involvement of the decarboxylation branch forming acyl-CoAs in the observed increase of aroma compounds in L-isoleucine fed strawberries could be even higher if considering increases in the amounts of ethyl 2-methyl-2-butenoate and 2-methyl-2-butenyl acetate. Synthesis of these esters seems to be consistent with a metabolic pathway where L-isoleucine is metabolized to 2-methylbutanoic acid, oxidized to 2-methyl-2-butenoic acid, and then further reduced to 2-methyl-2-butenol competing with direct esterification to 2-methyl-2-butenoates. Formation of a double bond at carbon 2 of 2-methylbutanoic acid structure could be catalyzed by an enzyme similar to the 2-methyl-branched chain acyl-CoA dehydrogenase. This enzyme has been purified from rat liver mitochondria and converts 2-methylbutanoyl-CoA to 2-methyl-2-butenoyl-CoA (*27*).

Data obtained in this study suggest that substrate concentration might limit ester biogenesis in strawberry, as amino acid addition to the culture media increased ester production, and the level of AAT activity was high enough for ester synthesis. Results indicate that the levels of branched ester moieties in strawberry, and perhaps ethyl moieties if considering alanine entering in the amino acid metabolic pathway, would depend on the relative activities of the pair 2-oxoacid decarboxylase/dehydrogenase type enzymes in the fruit. These relative activities could be affected by cultivar, maturity stage, and even environmental conditions, either on or off the plant. Recent studies have demonstrated that cultivar variations in two key aroma enzymes, alcohol dehydrogenase (ADH) and pyruvate dehy-

drogenase (PDC), could explain the different susceptibility of strawberry varieties to postharvest disorders such as off-flavor development during modified atmosphere storage (*28*, *29*). Interestingly, strawberry cultivars tolerant to high CO_2 produced less ethanol and acetaldehyde than nontolerant cultivars despite the fact that ADH and PDC activity levels were higher. Besides, tolerant cultivars accumulate more succinate, so that it may reflect lower carbon flux through the fermentation pathway and more via the TCA cycle. This phenomenon could be due to a CO_2-induced inhibition of succinate dehydrogenase from this cycle or a higher pyruvate dehydrogenase activity. Thus, as said before, activity levels of the pair pyruvate decarboxylase/dehydrogenase could be involved in the different distribution of aroma volatiles among strawberry cultivars. Although ADH activity is very important for aroma biosynthesis, it seems not to be a limiting factor. Mitchell and Jelenkovic (*30*) observed the presence of two NADPH- and NADH-dependent ADHs in strawberry receptacle with broad substrate specificities. NADH-dependent ADH showed higher activity against branched alcohols and nonaromatic aldehydes and an increase in activity during ripening, which is when characteristic aroma is produced. Similarly, Wyllie et al. (*31*) stated that banana ADH does not show the substrate specificity required to explain the composition of the branched chain esters present in this fruit aroma.

Data from this and other studies strengthen the hypothesis that biosynthesis of branched esters in strawberry aroma, and probably ethyl esters, is derived from amino acid metabolism, with the aminotransferase and especially the decarboxylating steps being critical for the release of esters precursors, as suggested in banana (*31*, *32*), before the last step of ester biosynthesis catalyzed by AAT.

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