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Cultivation Conditions Change Aroma Volatiles of Strawberry Fruit

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Abstract: Volatile compounds principally contribute to flavor of strawberry (*Fragaria* × *ananassa*) fruit. Besides to genetics, cultivation conditions play an important role in fruit volatile formation. Compared to soil culture as control, effects of substrate culture on volatile compounds of two strawberry cultivars ('Amaou' and 'Yuexin') were investigated. GC-MS analysis revealed significant difference in volatile contents of 'Amaou' strawberry caused by substrate culture. No significant effect was observed for cultivar 'Yuexin'. For 'Amaou' strawberry from soil culture produced higher volatile contents compared with substrate culture. This difference is contributed by high contents of esters, lactones, ketones, aldehydes, terpenes, hydrocarbons, acids, furans and phenols in 'Amaou' strawberry fruit from soil culture. Furanones, beta-linalool, trans-Nerolidol and esters are major contributor to strawberry aroma, whose contents are higher in soil culture planted fruit when compared to substrate culture. Moreover, strawberry fruit from soil culture had higher transcripts related to volatile biosynthesis were observed, including *FaQR*, *FaOMT*, *FaNES1*, *FaSAAT* and *FaAAT2*.

Keywords: strawberry; aroma volatiles; substrate culture; soil culture

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1. Introduction

Cultivated strawberry (*Fragaria* × *ananassa*), a genus of the Rosaceae, is an economically important berry fruit crop worldwide, and is valued for the nutritional quality and unique fragrance. Aroma compounds markedly contribute to fruit flavor, and directly affect the commercial quality of strawberry. The aroma compounds are associated with consumer liking and overall preference [1]. Although volatile compounds only account for 0.001 to 0.01 percent of a strawberry fruit weight, the slight changes can dramatically affect the fruit taste [1].

More than 360 different volatiles detected in strawberry [2], including terpenes, esters, lactones, furans, alcohols, aromatic hydrocarbons, organic acids, ketones, ethers and phenols [3]. Nevertheless, only about 20 volatiles contribute significantly to strawberry fruit flavor [4]. Volatile ester is major source of strawberry floral and fruity flavor [2], including methyl hexanoate, methyl butanoate, ethyl butanoate and ethyl hexanoate. Meanwhile, some low-content volatile compounds contribute markedly to the strawberry characteristic flavors [5]. The characteristic volatile compounds in strawberry include

furanone and terpenes [1]. Furanones were described as a caramel-like, sweet, fruity and floral aroma. The content of volatile furanones in strawberry is relatively low. However, furanones have a significant effect on fruit flavor compared to the threshold values [6], which mainly include 4-hydroxy-2,5-dimethyl-3(2)H-furanone (HDMF) and 2,5-dimethyl-4-methoxy-3(2)H-furanone (DMMF).

The type and quantity of volatile compounds contributing to fruit flavor vary between cultivars and maturities. A 35-fold difference in the content of volatiles evolved from different strawberry cultivars [4]. In addition to genetic factors, environmental factors, cultivation conditions and storage methods can also affect fruit aroma. For example, contents of volatile esters and furanones increased gradually with increasing temperature [7,8]. Light treatment affected furanones, esters and terpenoids in strawberry fruits during postharvest storage [4]. When the internal atmosphere reached O_2 concentrations below 2% and CO_2 above 25%, strawberry fruit emitted an unusual flavor pattern, in which ethyl acetate and methyl acetate were the main compounds [9]. Moreover, cultivation conditions also significantly changed aroma volatiles in strawberry. Under the rain cover cultivation, synthesis of esters in strawberry fruits decreased [10]. By increasing the nitrogen supply concentration in the hydroponic environment, cultivated strawberries could produce more esters flavor [11]. Strawberry fruits were tradionally planted in soil, recently substrate culture is applied with fast rise in greenhouse numbers. However, effect of substrate culture on strawberry fruit volatiles remains unknown.

Genes related to volatile synthesis have been identified in strawberry. FaQR (quinone oxidoreductase) and FaOMT (O-methyltransferase) were identified as the key enzymes in furanone biosynthesis. The 4-hydroxy-5-methyl-2-methylene-3(2*H*)-furanone can be catalyzed by FaQR (strawberry quinone oxidoreductase) to produce the aroma-active HDMF [12,13]. Then FaOMT (O-methyltransferase) subsequently catalyzes the methylation of HDMF to form DMMF [14]. Additionally, HDMF also can be catalyzed to 2,5-dimethyl-4-hydroxy-2H-furan-3-one glucoside (HDMF-glucoside) by UGT71K3 (a UDP-dependent glycosyltransferase), and further catalyzed to 2,5-dimethyl-4-hydroxy-2H-furan-3-one 6′-O-malonyl-β-d-glucopyranoside (HDMF malonyl-glucoside) during late stages of fruit ripening [15]. The characteristic terpenoids in cultivated strawberry are trans-Nerolidol and beta-linalool [16]. FaNES1 (nerolidol synthase) and FvPINS (pinene synthase) were characterized to be involved in terpene biosynthesis in cultivated and wild strawberries respectively [16]. The most vital esters in cultivated strawberry are ethyl butanoate, 2-methylbutanoate, ethyl hexanoate and methyl butanoate. Octyl acetate, carveyl acetate, methyl nicotinate, butyl formate, methyl N-formylanthranilate, decyl butanoate, decyl acetate and methyl anthranilate were only found in wild strawberry [17]. The last crucial step in the biosynthesis of strawberry volatiles is the esterification reaction of alcohols with an acyl moiety of acyl-CoA catalyzed by FaAATs (alcohol acyltransferase). FaSAAT and FaAAT2 were identified to be involved in the biosynthesis of esters [18,19]. These charcaterized genes are essential to investigate effects of substrate on volatile synthesis in strawberry.

In this study, two cultivars of strawberry 'Amaou' (*Fragaria* × *ananassa*) and 'Yuexin' (*Fragaria* × *ananassa*) were planted under substrate and soil cultivation conditions. Aroma volatiles of strawberry fruits were analyzed using headspace solid phase microextraction (HS-SPME) combined with GC-MS detection. Contents of volatiles produced by strawberry cultivated from two different methods were compared. Furthermore, expression patterns of genes related to important volatile compounds, including furanone, terpene and ester, were analyzed using qPCR. These results will provide knowledge for effects of cultivation conditions on aroma volatiles of strawberry fruit.

2. Materials and Methods

2.1. Plant Materials

Two octoploid cultivars 'Yuexin' and 'Amaou' (*Fragaria* × *ananassa*) were planted in the orchard of Zhejiang Academy of Agricultural Sciences in Haining, Zhejiang province,

China. The formula in substrate culture includes 40% peat, 20% organic medium, 10% vermiculite, 20% basic fertilizer, 10% perlite, slow-release fertilizers 2 kg/m^3, bactericides and stabilizer (Hangzhou Jinhai Agricultural Science and Technology Co., LTD, Hanghzou, China). Strawberries in substrate and soil culture were grown in the same greenhouses. Strawberry fruit harvested from soil culture were used as control. Fruits at four developmental stages, G (green), T (turning), IR (intermediate red), and R (full red) were harvested, then transported to the laboratory within 1.5 h. Six fruits with uniform size and free of visible defects were used as a biological replicate. For each stage, three biological replicates were sampled. Fruits were divided into the apical and basal sections after removing the calyces, and were then sampled separately according to our previous study [20]. Samples were rapidly cut into pieces and immediately frozen in liquid nitrogen, then stored at −80 °C for further use.

2.2. RT-qPCR Analysis

Total RNA from strawberry fruit samples was extracted using the CTAB method [20]. Potentail genomic DNA contamination was eliminated using gDNA Eraser (Ambion). The concentration and purity of RNA used for cDNA synthesis were determined by spectrophotometry, and the quality of RNA were also examined by agarose gel electrophoresis. A total of 1000 ng DNase-treated RNA was used for reverse transcription to generate cDNA by PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology, Dalian, China). The resulting cDNA products were diluted for RT-qPCR analysis.

The RT-qPCR reactions were carried out in a total volume of 20 µL, containing 2 µL of diluted cDNA template, 2 µL of gene-specific primers (1.0 µM), 6 µL of sterile distilled water and 10 µL of PCR SYBR supermix (Bio-Rad, Hercules, CA, USA) on a CFX96 real time-PCR machine (Bio-Rad, Hercules, CA, USA). Strawberry *GAPDH* gene (GenBank Accession number: AB363963.1) and the interspacer 26S-18S strawberry RNA housekeeping gene *FaRIB413* (Accession number: gene33863) were used as internal reference genes [20,21]. Target gene expression levels were normalized against the geometric mean of the two reference genes. Gene-specific primers were designed using Primer Premier 6.0 software. The melting curves of each pair of primers and negative control were used to evaluate the specificity of primers. To further confirm the specifity of primers, RT-qPCR products were checked by agarose gel electrophoresis and sequenced. Accession numbers and primers of all genes used in RT-qPCR analysis were listed in Tables S1 and S2. RT-qPCR data was analyzed as previously described using the $2^{-\Delta\Delta CT}$ method [22]. Three independent biological replications were carried out for each assay.

2.3. Detection of Volatile Compounds in Strawberry Fruits

An automated headspace solid-phase microextraction (HS-SPME) method was used to detect DMMF and other volatile compounds in strawberry fruits [23]. In the vial, 0.5 g powdered samples were treated with 1 mL EDTA solution (100 mM, pH 7.5), 1 mL 20% (m/v) $CaCl_2$ solution and 20 µL 2-octanol (0.07 µg/µL) as the internal standard. The vial was homogenized, then placed on the sample tray for subsequent analysis by a CombiPAL autosampler (CTC Analytic, Zwingen, Switzerland). The volatiles were sampled using the headspace solid-phase microextraction with a 65 µm polydimethylsiloxane divinylbenzene fiber. Firstly, the vial was incubated for 10 min at 50 °C at 500 rpm, then volatiles were extracted for 30 min under continuous agitation at the same temperature. Finally, the fiber was desorbed in the GC injection port in splitless mode for 5 min. Subsequent analysis was performed using the 7890A GC-MS system (Agilent Technologies, Santa Clara, CA, USA; J&W Scientific, Folsom, CA, USA) equipped with a DB-5 ms column (60 m × 250 µm × 1 µm; J&W Scientific). The helium was used as the carrier gas and the flow rate was 1.2 mL/min. The gas chromatography instrument was programmed at 35 °C for 2 min, then increased to 250 °C at a rate of 5 °C per minute. The injection port, MS source, and interface temperatures were 250 °C, 230 °C, and 260 °C, respectively. The ionization potential in the 5975B mass spectrometer device was 70 eV, and the scanning

speed was seven scans per second. Volatile compounds were identified by comparing retention time of authorized standards available, and electron ionization mass spectra with the NIST/EPA/NIH Mass spectrometry library (NIST-08 and Flavor). Volatile compounds were calculated quantitatively using the peak area of the internal standard 2-octanol (0.07 μg/μL) as a reference based on total ion chromatogram (TIC). DMMF, beta-linalool and trans-Nerolidol was identified by comparison with injected standard (Sigma-Aldrich, St. Louis, MI, USA).

A liquid-injection system equipped with CTC Pal ALS was carried out for detecting the HDMF [20]. Fruit samples were ground in liquid nitrogen, and approximately 1 g powdered samples were treated with 2 mL 20% (m/v) NaCl in a 10 mL tube. Then 300 μL CH_2Cl_2 and 20 μL 0.766 μg/μL 2-octanol as the internal standard were added into each tube. The mixture was homogenized and stored at room temperature for 20 min, then centrifuged for 5 min at 12,000 rpm. The subnatant was transferred to a new 1.5 mL tube containing 20 mg anhydrous sodium sulfate, then left at room temperature for 30 min without shaking. Ultimately 150 μL solution was transferred to a GC vial, then placed on the sample tray. 1 μL samples were injected by a CombiPAL autosampler (CTC Analytic), and the further analysis was performed using a 7890A GC-MS system (Agilent Technologies, J&W Scientific) equipped with a DB-WAX column (30 m × 250 μm × 0.25 μm; J&W Scientific). The carrier gas was helium (1.2 mL/min). The MS source, injection port (splitless injection mode), and interface temperatures were 230 °C, 250 °C and 260 °C respectively. The oven temperature was set at 60 °C for 4 min, then increased to 180 °C at a rate of 4 °C per minute and maintained for 30 min finally. HDMF was identified by comparison with injected standard (Sigma-Aldrich: 40703). Based on total ion chromatogram, the content of HDMF was calculated quantitatively using the peak area of the internal standard 2-octanol as a reference. The classification of strawberry aromatic substances was prepared according to previous studies [24,25].

2.4. Statistical Analysis

Contents of volatiles detected were log2-transformed to generate heat map by Multi-Experiment Viewer software 4.9.1. Data analysis was performed using the Microsoft Excel (version 2016), and Figures were prepared with Sigmaplot 10.0 (Systat, San Jose, CA, USA). One-way ANOVA was carried out to assess significant differences using SPSS Statistics software (version 22.0). Significance is indicated by * ($0.01 < p < 0.05$) and ** ($p < 0.01$). Student's *t* test was used for significance analysis of the data in Tables 1 and 2.

Table 1. Content of aroma volatiles (μg/g FW) in apical section of 'Amaou' strawberry.

Volatiles	Culture Conditions	G *	T	IR	R
Esters	Soil	0.073 ^{a}	0.124 ^{a}	0.250 ^{a}	0.764 ^{a}
	Substrate	0.013 ^{b}	0.035 ^{b}	0.095 ^{b}	0.507 ^{b}
Lactones	Soil	0.000 ^{a}	0.024 ^{a}	0.231 ^{a}	0.923 ^{a}
	Substrate	0.000 ^{a}	0.000 ^{b}	0.089 ^{b}	0.415 ^{b}
Ketones	Soil	0.288 ^{a}	0.305 ^{a}	0.136 ^{a}	0.158 ^{a}
	Substrate	0.159 ^{b}	0.136 ^{b}	0.138 ^{a}	0.128 ^{b}
Alcohols	Soil	0.042 ^{a}	0.031 ^{a}	0.028 ^{a}	0.008 ^{a}
	Substrate	0.002 ^{b}	0.102 ^{b}	0.037 ^{a}	0.007 ^{a}
Aldehydes	Soil	2.340 ^{a}	2.246 ^{a}	1.954 ^{a}	1.665 ^{a}
	Substrate	2.475 ^{a}	1.606 ^{b}	1.524 ^{b}	1.480 ^{a}
Terpenes	Soil	0.388 ^{a}	0.572 ^{a}	4.439 ^{a}	12.006 ^{a}
	Substrate	0.490 ^{b}	0.658 ^{a}	1.156 ^{b}	9.280 ^{b}
Hydrocarbons	Soil	0.085 ^{a}	0.117 ^{a}	0.158 ^{a}	0.189 ^{a}
	Substrate	0.018 ^{b}	0.030 ^{b}	0.031 ^{b}	0.047 ^{b}

Table 1. *Cont.*

Volatiles	Culture Conditions	G *	T	IR	R
Acids	Soil	0.100 [a]	0.013 [a]	0.060 [a]	0.150 [a]
	Substrate	0.010 [b]	0.008 [a]	0.009 [b]	0.102 [b]
Furans	Soil	0.005 [a]	0.003 [a]	0.004 [a]	0.126 [a]
	Substrate	0.013 [a]	ud [b]	ud [b]	0.065 [b]
Ethers	Soil	ud [#,a]	0.002 [a]	ud [a]	ud [a]
	Substrate	ud [a]	0.001 [a]	0.001 [b]	0.002 [b]
Phenols	Soil	0.069 [a]	0.078 [a]	0.088 [a]	ud [a]
	Substrate	0.035 [b]	0.050 [b]	ud [b]	ud [a]

* G, T, IR and R represents different fruit development stages, corresponding to green, turning, intermediate red and full red, respectively. # ud, under detected limits. Student's *t* test was carried out using SPSS Statistics software 22.0. Significant analysis was carried out on the fruit aroma under substrate and soil cultivation at the same stage. Different letters indicate statistically significant differences ($p < 0.05$).

Table 2. Content of aroma volatiles (μg/g FW) in basal section of 'Amaou' strawberry.

Volatiles	Culture Conditions	G *	T	IR	R
Esters	Soil	0.066 [a]	0.109 [a]	0.137 [a]	0.690 [a]
	Substrate	0.005 [b]	0.028 [b]	0.064 [b]	0.398 [b]
Lactones	Soil	0.020 [a]	0.006 [a]	0.118 [a]	0.739 [a]
	Substrate	0.000 [b]	0.002 [a]	0.023 [b]	0.273 [b]
Ketones	Soil	0.308 [a]	0.319 [a]	0.154 [a]	0.165 [a]
	Substrate	0.131 [b]	0.136 [b]	0.143 [a]	0.120 [a]
Alcohols	Soil	0.040 [a]	0.017 [a]	0.018 [a]	0.000 [a]
	Substrate	0.156 [b]	0.075 [b]	0.039 [b]	0.000 [a]
Aldehydes	Soil	2.050 [a]	1.808 [a]	1.673 [a]	1.536 [a]
	Substrate	1.636 [b]	1.397 [b]	1.445 [a]	1.357 [b]
Terpenes	Soil	0.627 [a]	0.830 [a]	2.939 [a]	9.397 [a]
	Substrate	0.182 [b]	0.309 [b]	1.025 [b]	4.458 [b]
Hydrocarbons	Soil	0.174 [a]	0.197 [a]	0.239 [a]	0.182 [a]
	Substrate	0.026 [b]	0.060 [b]	0.041 [b]	0.055 [b]
Acids	Soil	0.026 [a]	0.023 [a]	0.014 [a]	0.062 [a]
	Substrate	0.002 [b]	0.004 [b]	0.016 [a]	0.017 [b]
Furans	Soil	0.003 [a]	ud [#,a]	0.002 [a]	0.037 [a]
	Substrate	ud [b]	ud [a]	ud [b]	0.018 [b]
Ethers	Soil	0.002 [a]	0.004 [a]	ud [a]	0.002 [a]
	Substrate	ud [b]	0.002 [a]	0.004 [b]	0.002 [a]
Phenols	Soil	0.111 [a]	0.118 [a]	0.258 [a]	0.065 [a]
	Substrate	0.040 [b]	0.098 [a]	0.112 [b]	0.049 [a]

* G, T, IR and R represents different fruit development stages, corresponding to green, turning, intermediate red and full red, respectively. # ud, under detected limits. Student's *t* test was carried out using SPSS Statistics software 22.0. Significant analysis was carried out on the fruit aroma under substrate and soil cultivation at the same stage. Different letters indicate statistically significant differences ($p < 0.05$).

3. Results

3.1. Effect of Substrate Culture on Volatile Contents of Strawberry Fruits

The total aroma components of strawberry fruits at different developmental stages in two cultivars ('Amaou' and 'Yuexin') under substrate and soil culture was analyzed (Figure 1). A total of 163 volatiles were detected in strawberry fruits by employing GC-MS, including 53 esters, 34 aldehydes, 16 terpenes, 8 alcohols and other compounds (Table S3). In general, contents of volatiles tend to increase with fruit ripening in both basal and apical

sections. For fruit harvested at R stage, higher contents of volatiles were detected in the apical section. Compared to 'Yuexin', 'Amaou' strawberry fruit produced significantly higher contents of volatiles during development and ripening (Figure S1).

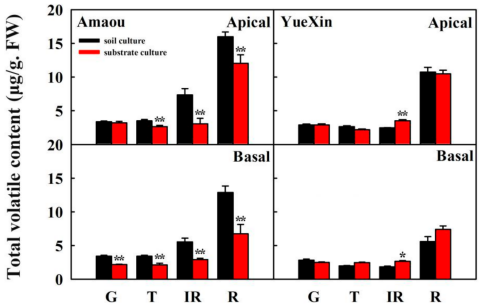

Figure 1. Effects of substrate and soil culture on the fruit total aroma components in two strawberry cultivars. G, T, IR and R represents different fruit development stages, corresponding to green, turning, intermediate red and full red, respectively. Bars indicate means ± SD from three biological replicates. Asterisks indicate significant differences (* $p < 0.05$; ** $p < 0.01$).

Significant difference in volatile contents were observed for 'Amaou' strawberry between two different cultivation conditions. Strawberry harvested from soil culture produced significant higher contents of volatiles than substrate culture. For fruit tissues from the basal section at R stage, contents of volatiles were 12.87 μg/g and 6.75 μg/g under soil and substrate culture, respectively (Figure 1). Contents in substrate culture were reduced by about 47.57% in the basal section when 'Amaou' fruit at R stage. Significant reduction of volatiles were also observed in the apical section of strawberry sampled from substrate culture when compared to soil culture. However, no significant difference in volatile contents were observed for 'Yuexin' strawberry between these two cultivation patterns. In contrast, 'Yuexin' strawberry from substrate culture produced slightly more volatiles at IR stage. These results indicated that substrate culture affect contents of 'Amaou' strawberry during fruit ripening. We next investigated changes in contents of chemical classes and individual volatiles in 'Amaou' strawberry fruits.

3.2. Changes in Volatile Chemicals in 'Amaou' Strawberry Fruits under Substrate and Soil Culture Conditions

In order to determine the specific volatile compounds that were affected by substrate culture, we analyzed profiles of various kinds of volatiles, including esters, lactones, ketones, alcohols, aldehydes, terpenes, hydrocarbons, acids, furans, ethers and phenols (Figure 2). A considerable accumulation of esters, lactones, terpenes and furans was observed in both basal and apical sections, but these volatiles are more abundant in the apical sections of fruits. Content of esters, lactones, ketones, aldehydes, terpenes, hydrocarbons, acids, furans and phenols were reduced by substrate culture compared to soil conditions, especially in the IR and R stage. Compared with substrate culture, contents of total esters were approximately 50.80% and 73.35% higher at R stage under soil culture for apical and basal parts, respectively (Tables 1 and 2). In the case of terpenes, concentrations with soil culture at R stage were approximately 110.80% higher than that in basal section of fruit harvested from substrate conditions.

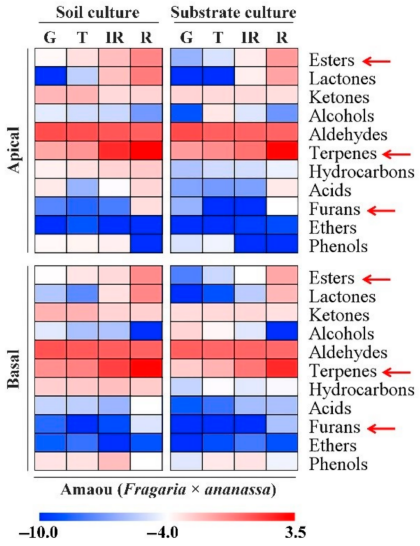

Figure 2. Heatmap display of aroma volatiles in 'Amaou' strawberry. G, T, IR and R represent green, turning, intermediate red and full red, respectively.

3.3. Changes in Contents of Furanone and Expression of Synthesis-Related Gene Expression Caused by Substrate Culture in 'Amaou' Strawberry

Furanone contributes to the characteristic aroma of strawberry fruits, we further analyzed effects of substrate culture on furanone content and expression levels of furanone biosynthetic genes during fruit ripening. HDMF and DMMF are two major furanones detected in strawberry fruit. During fruit ripening, contents of HDMF and DMMF could be detected at IR stage, and then rapidly accumulated thereafter (Figure 3A). Higher contents of these two chemicals were observed in apical section than basal section. In general, contents of HDMF and DMMF were more abundant under soil culture than substrate culture.

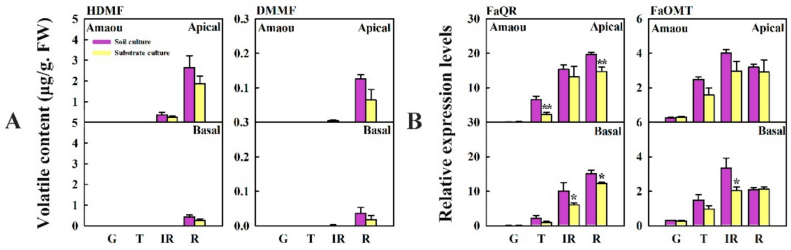

Figure 3. Analysis of furanone content (**A**) and expression of *FaQR* and *FaOMT* (**B**) in 'Amaou' strawberry fruits. Bars stand for means ± SD from three biological replicates. Asterisks indicate significant differences (* $p < 0.05$; ** $p < 0.01$).

FaQR and *FaOMT* genes were identified as the key genes for HDMF and DMMF biosynthesis, respectively [12,23]. Transcript levels of *FaQR* accumulated dramatically with fruit ripening (Figure 3B). For fruit at R stage, significantly higher expression levels of *FaQR* were observed in the apical section compared to the basal section. Abundance of *FaOMT* transcripts increased during fruit ripening, with little difference between the two sections at the R stage. From G to R stage, *FaQR* and *FaOMT* gene expression levels in soil culture were higher than substrate culture generally. These results indicated that soil culture planted strawberry fruit produced more furanone content, accompanying with higher *FaQR* and *FaOMT* gene expression in 'Amaou' fruits compared to substrate culture.

3.4. Changes in Content of Beta-Linalool and Trans-Nerolidol and Expression of FaNES1 Caused by Substrate Culture in 'Amaou' Strawberry

Beta-linalool and trans-Nerolidol are the main volatile terpenes in cultivated strawberry fruits [16]. Contents of beta-linalool were below the limit of detection until fruits reached the R stage (Figure 4A). Trans-Nerolidol accumulated mainly at IR and R stages in fruits, reaching the highest at R stage. Strawberry fruit grown in soil culture produced more beta-linalool and trans-Nerolidol when compared to substrate culture. *FaNES1* expression increased along strawberry fruit ripening (Figure 4B). More abundance of transcript levels were observed in the apical sections compared to the basal sections at the T and R stages. Fruit from soil culture had higher *FaNES1* gene expression levels at T and IR stages when compared with substrate culture. Therefore, changes in expression pattern of FaNES1 were similar with profiles of beta-linalool and trans-Nerolidol throughout fruit development and ripening. Strawberry fruit from soil culture had a slight promoting effect on beta-linalool and trans-Nerolidol biosynthesis in 'Amaou' strawberry cultivar.

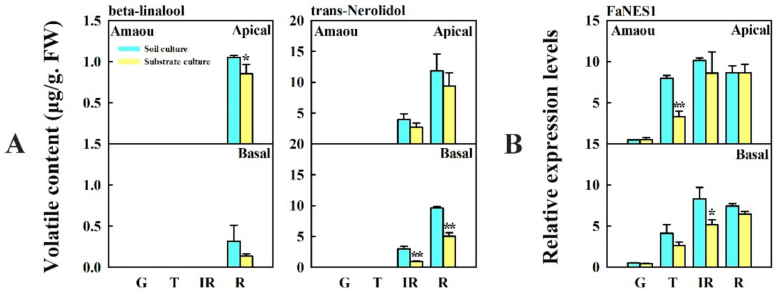

Figure 4. Changes in contents of beta-linalool and trans-Nerolidol (**A**) and expression of *FaNES1* (**B**) in 'Amaou' strawberry fruits. Bars stand for means ± SD from three biological replicates. Asterisks indicate significant differences (* $0.01 < p < 0.05$; ** $p < 0.01$).

3.5. Effect of Substrate Culture on Volatile Esters and Expression of FaSAAT and FaAAT2 in 'Amaou' Strawberry

Volatile esters were detected at all stages during fruit development and ripening, and accumulated gradually with fruit ripening (Figure 5A). From the G to the R stage, compared with substrate culture, significantly higher contents were observed for volatile esters in 'Amaou' strawberry fruits cultivated in soil. Two alcohol acyltransferases FaSAAT and FaAAT2 have been identified to be involved in the biosynthesis of volatile esters [18,19]. To investigate whether difference under soil and substrate culture was associated with difference in the expression levels of these two biosynthetic genes, transcription levels of *FaSAAT* and *FaAAT2* in fruits were analyzed (Figure 5B). For *FaSAAT*, the soil culture showed significantly higher expression levels at T stage in apical section. For basal section, soil culture fruit at IR stage had higher transcripts of *FaSAAT* relative to substrate culture. Transcript levels of *FaAAT2* increased as strawberry fruit developed. Higher levels of *FaAAT2* transcripts were observed in soil culture compared to the substrate culture. These results show that soil cultivation contributed to accumulation of esters in 'Amaou' strawberry.

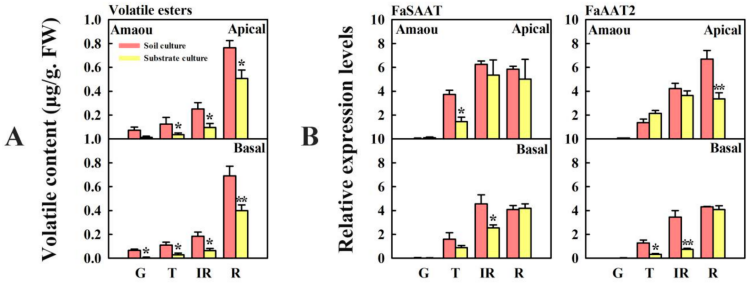

Figure 5. Changes in volatile esters (**A**) and expression of *FaSAAT* and *FaAAT2* (**B**) in 'Amaou' strawberry. Bars stand for means ± SD from three biological replicates. Asterisks indicate significant differences (* $0.01 < p < 0.05$; ** $p < 0.01$).

4. Discussion

This study analyzed changes of volatile compounds in two cultivated strawberry fruits under substrate and soil culture. In general, contents of aroma volatiles from 'Amaou' were significantly higher than 'Yuexin' during strawberry development and ripening. Considering it is rich in volatile compounds, we suspected that contents of volatiles in 'Amaou' strawberry may be more susceptible to external cultivation conditions. As expected, the present study showed that the cultivation methods significantly changed the contents of strawberry cultivar 'Amaou'. Compared to the substrate cultivation method, soil cultivation contributed to producing higher contents of esters, lactones, ketones, aldehydes, terpenes, hydrocarbons, acids, furans, and phenols in 'Amaou' strawberry. In contrast, for the strawberry cultivar 'Yuexin' with less aroma intensity, different cultivation methods had little effect on fruit aroma volatiles. These results indicate that strawberry fruit with higher contents of volatiles were more sensitive to cultivation practices. Changes in aroma volatile profiles due to cultivation methods are affected by strawberry cultivar.

The present study showed significantly higher contents of volatiles were observed in strawberry fruit cultivated in soil rather than substrate. For example, contents of esters produced by substrate cultivation were reduced by approximately 30% and 40% at ripe (R) stages for apical and basal tissues, respectively. Volatile esters contribute to fruity aroma of multiple ripe fruits, including strawberry. It seems that soil culture was conducive to the formation of the aroma quality of strawberry fruits during the development and ripening stages. This difference in content of volatiles, particular for fruity esters, may be associated with difference in water-holding capacity between soil and substrate. In the present study, clayed soils were used for strawberry planting. Clayed soils have better water retention capacity and volumetric wetness than substrate. For grape, fruit from clayed soils had greater sweet and fruity aroma than that from sandy soils [26]. In addition, the influence of rain cover cultivation on aroma quality of strawberries has been reported [10]. Secondary differences in nutrition compositions may contribute to differences in aroma volatiles between different cultivation methods. For example, nitrogen fertilization of a vineyard increased the contents of aroma esters of grape and wines [26]. Moreover, complex additives in substrate—such as rye malt, corn husk, wheat bran and soy flour—altered mineral compositions which, in turn, influenced volatile compounds in mushroom [27]. Thirdly, microbial composition difference may contribute changes in volatile compounds of strawberry under different cultivation methods. Volatile compounds are sensitive to environmental factors, including abiotic and biotic stresses. Changes in volatiles caused by infection of bacterial were widely observed in plants and fruits. Overall, great impacts of cultivation conditions on fruit aroma volatile profiles have been reported in multiple fruit species, including grape, peach and strawberry [28–34]. For instance, organic strawberries are sweeter, taste better, and have greater overall acceptance and appearance than conventional strawberries [34]. It would be interesting to investigate microbial compositions and mineral compositions in soil and substrate in future. This observation will shed light on effects of cultivation methods on aroma volatiles of strawberry fruit.

Considering the vital influence of volatiles on fruit flavor, a better understanding of the impact of substrate and soil cultivation on strawberry aroma will allow growers to produce better strawberries. Furthermore, growers can adjust the selection of strawberry cultivars under different cultivation methods to produce fruits with better aroma quality. Producers can choose the most suitable cultivation patterns and adjust the type of soil according to the quality of fruit they need. Given that less influence was observed for changes in aroma volatiles, growers can choose strawberry cultivars with less aroma intensity for viaduct cultivation, which would be suitable for plant factory cultivation.

In summary, different cultivation methods can affect the formation of aroma compounds in strawberry fruits. The effect of substrate cultivation methods on 'Amaou' strawberry was greater than for 'Yuexin'. For the strawberry cultivar 'Amaou', soil culture is recommended due to its contribution to accumulation of aroma compounds during fruit development and ripening. Although differences in the expression of genes related to volatile synthesis were observed for strawberry fruit, caused by substrate culture, more experiments such as on epigenetic factors are required to further clarify the molecular mechanism.

5. Conclusions

Different cultivation methods can affect the formation of aroma compounds in strawberry fruits. Through the analysis of aroma content and aroma-associated gene expression in the fruit development and ripening stage under the substrate and soil culture condition, it was found that the effect of different cultivation methods on 'Amaou' strawberry was greater than 'Yuexin'. Soil culture can promote the accumulation of aroma compounds in cultivated strawberry 'Amaou' fruits.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7040081/s1, Table S1. Genes involved in volatiles biosynthesis in strawberry. Table S2. Primers used in RT-qPCR. Table S3. Volatile compounds detected in strawberry fruit using GC-MS analysis. Figure S1. Volatiles of 'Amaou' and 'Yuexin' under soil cultivation conditions.

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