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AROMA QUALITY OF FRUITS OF WILD AND CULTIVATED STRAWBERRY (*FRAGARIA* SPP.) IN RELATION TO THE FLAVOUR-RELATED GENE EXPRESSION

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ABSTRACT

Expression profiles of flavour-related genes and the aroma quality of fruit headspace were investigated in the four strawberry genotypes 'Reine des Vallées' (*Fragaria vesca*), 'Profumata di Tortona' (*F. moschata*), 'Onda' and VR 177 selection (*F.* × *ananassa*). Differences in the expression level of genes coding of strawberry alcohol acyltransferase (*SAAT*), *F.* × *ananassa* nerolidol synthase 1 (*FaNES1*) and *F. vesca* monoterpene and sesquiterpene synthases (*FvPINS* and *PINS1*, respectively) were detected among these genotypes. In fruits of *F.* × *ananassa* the terpenoid profile was dominated by nerolidol, whereas wild species produced mainly monoterpenes. It was correlated with the higher induction of *FaNES1* in cultivated and *PINS* gene in the wild *Fragaria* species. The flavour biogenesis in ripening fruits was determined by the expression of *SAAT* gene, especially visible for 'Profumata di Tortona' and 'Onda' strawberries. The fruit solid-phase microextraction (SPME) headspace was analysed using the Gas Chromatography-Olfactometry (GC-O), that allows for the chromatographic separation of volatiles together with their olfactometric evaluation. 'Reine des Vallées' fruits have a peculiar profile characterized by high concentrations of limonene, linalool and mesifurane that resulted in "spiced", "citrus, floral" and "sweet, baked" descriptors. The character impact compound in 'Profumata di Tortona' fruits was ethyl butanoate, responsible for "sweet" and "fruity, strawberry" descriptors. However, it was detected in lower amount in comparison to the data obtained for *F.* × *ananassa* strawberries. The sesquiterpene nerolidol was identified in both cultivated strawberry genotypes.

Key words: fruit, aroma, quality, olfactometry

INTRODUCTION

Strawberries are known as a source of phytochemicals with health promoting properties: phenolic compounds, such as anthocyanins and ellagitannins, are widely recognized as antioxidants (Aabi et al. 2012). In parallel, consumers' satisfaction depends upon good eating quality resulting from flavour, taste and aroma (Pelayo et al. 2003). Therefore, nowadays the improvement of flavour and aroma profiles is taken into account in the breeding programmes (Marta et al. 2004; Noguchi et al. 2002; Jones 1966). In this aspect, wild strawberries were recognised as interesting donors of many genes responsible for the traits desired by consumers (Ulrich et al. 2007). The aroma of ripening strawberry is composed by volatiles belonging to several chemical classes. The main components are esters. Among them, ethyl 2-methylbutanoate, methyl and ethyl butanoate, ethyl hexanoate and hexyl acetate are the most important flavour-active components. They are providing the "sweet–fruity" odour note (Ménager et al. 2004), along with aldehydes, alcohols, furans and sulphur compounds

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(Douillard & Guichard 1989; Rizzolo et al. 1995; Forney et al. 1996; Azodanlau et al. 2003, 2004). Other volatiles, such as the monoterpene linalool, γ-dodecalactone and some sulphur compounds, are the most important contributors to strawberry aroma (Schieberle & Hoffmann 1997) along with few impact compounds, such as furaneol (2,5-dimethyl-4-hydroxy-3(2H)-furanone) and its methyl ether (Zabetakis & Holden 1997; Polesello et al. 1993).

In order to perform the olfactometric evaluation of food aroma, the choice of an adequate extraction method is a critical issue that largely contributes to the reliability of olfactory results. The solid–phase microextraction is a solvent-free, rapid and inexpensive method for the isolation and the concentration of volatiles present in the headspace, without deterioration due to temperature or solvent effects (Lv et al. 2012). In recent years it has been widely used in combination with gas chromatography-olfactometry (GC-O) to study aroma-active compounds (Guillot et al. 2006; Villière et al. 2012).

This work summarizes the differences in olfactometric profiles and the expression patterns of genes that influence fruit flavour among selected strawberry genotypes belonging to *F.* × *ananassa*, *F. vesca* and *F. moschata.*

MATERIALS AND METHODS

Plant material

The study was carried out on genotypes of *F.* × *ananassa* ('Onda' and selection VR177), *F. vesca* ('Reine des Vallées', RdV) and *F. moschata* ('Profumata di Tortona', PdT). Plants were grown in Tortona (Piemont region), except for VR 177 which was cultivated in Martorano di Cesena (Emilia-Romagna region) in the experimental orchards of CRA. The strawberries were harvested at the fully-ripe stage, assessed visually on the basis of fruit colour. After harvest they were transported to the Food Technology research unit of the CRA in Milano, where fruit quality parameters and aroma profiles were assessed.

Quality parameters

Soluble solids content (SSC) and titratable acidity (TA) were measured on homogenized fruits. The results are the mean of three replicate samples. SSC (°Brix) was measured using a refractometer RFM 81 and TA (meq·100 g^{-1}) was assessed by means of a Dosimat 682 titroprocessor.

RNA extraction and real-time RT-PCR analysis

Total RNA was extracted from fruits (4 g) according to Chang et al. (1993) and purified with the RNA Clean up protocol (Qiagen, Valencia, CA, USA). Obtained RNA (1 μg per sample) was a template for cDNA synthesis following the iScript cDNA synthesis kit protocol (Bio-Rad). Single strand cDNA (20 ng) was used for real-time RT-PCR. The experiments were performed using the 2x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the CFX-96 device (Bio-Rad). Conditions of relative quantitative analysis were as follows: 94 °C for 10 min; 40 cycles of 94 °C for 15 s, 60 °C for 15 s and 72 °C for 20 s; and a melting curve from 58 to 95 °C at 0.5 °C increments. Three technical replicates were prepared for each tested sample. The expression of four fruit-flavour associated genes: strawberry alcohol acyltransferase (*SAAT: AF193789.1*), *F.* × *ananassa* nerolidol synthase 1 (*FaNES1: CAD57081.1*) and *F. vesca* monoterpene and sesquiterpene synthases (*FvPINS: AX529025.1* and *PINS1: AJ001452.1*, respectively) was determined. Gene-specific primers were designed within consecutive exons using Primer3 software (Table 1). Relative quantification was normalized to the housekeeping control gene (*rRNA 18S: X15590*). Gene expression was calculated by using $2^{-\Delta Ct}$ method, where ΔCt represents the difference between the Ct value of the target gene and the Ct value of the housekeeping gene (Schmittgen & Livack 2008).

Gas Chromatography-Olfactometry (GC-O)

The olfactometric analysis was carried out by 3 panellists aged between 24 and 43 years. Before the analysis of the samples all panellists attended two training sessions to identify the main odour categories. The following standards were used: γ-undecalactone (fruit, apricot), furaneol and mesifurane (strawberry, caramel), ethyl butanoate (fruit, apple), methyl disulphide (cabbage), (E)-2-hexenal (herbaceous, bug), hexanal (green), hexyl acetate (fruity, sweet), methyl 2-methylbutanoate (fruity), methyl hexanoate (fruity, fresh, sweet), methyl butanoate (ether, fruity, sweet), linalool (flower, lavender).

Table 1. Fruit-flavour associated genes identified in *Fragaria* spp. (*SAAT* coding alcohol acyltransferase; *FaNES1* coding nerolidol synthase 1; *FvPINS* coding monoterpene synthase; *PINS1* coding sesquiterpene synthase)

Gene and Accession Number		Primer Forward (5'→3')	Primer Reverse (3'→5')
SAAT	AF193789.1	ATGCCGTCACTGGTTTTCTC	GTGCCCACCAGAACAAGTTT
FaNES1	CAD57081.1	TGGGACGATTTAGGAAGTGC	TGAATGATGCTGGAAATGGA
FvPINS	AX529025.1	AGGAGCTGACAAAGCAAGGA	AAAGACACGACGGAAAGCAT
PINS1	AJ001452.1	TGAATACGGGGTTTCAGAGC	TCATCAGTTTTCCGACATGC
rRNA18S	X15590	ATTTCGGTCCTATTCTGTTGGC	GCTTTCGCAGTTGTTCGTCTTT

Sample preparation

Each sample consisted of 10 g of homogenized pulp (three replicates for each selection, each one consisting of a pool of 10 fruits) put in a 20 mL vial closed with an aluminium cap with silicone-rubber septum and stored at -30 °C until analysis. The extraction of volatile compounds was performed by headspace solid-phase microextraction (HS-SPME) using a DVB/CAR/PDMS fibre (absorption step: 40°C for 30 min; desorption step in the injector port: 250°C for 5 min in splitless). GC analyses were carried out with an Agilent 6890 N GC equipped with a FID and a DB-WAX capillary column (60 m× 0.25 mm I.D., 0.25 µm film thickness); injector and FID temperatures, 250 °C; column temperature program: 40 °C for 10 min, 4 °C min^{-1} to 220 °C held for 5 min. The gas chromatograph is linked to an olfactometric system that includes the Olfactory Detector Port ODP2 Gerstel (Gerstel GmbH) equipped with the ODPneumatics module to control humidification and make-up gas flows; the olfactometric data (intensity on a 5-point intensity scale where 0 = no odour and 4 = very intense odour, duration and area of each odour event, OE) are collected with the ODP-Recorder software. The area of each OE is calculated by the software from the intensity and duration values and is shown as a chromatographic peak. The compounds were identified by comparison with linear retention index of standards and by their odour and expressed as µg of hexyl acetate or α-terpineol equivalents 100 g^{-1} fresh weight.

Data analysis

Statistical analyses were carried out with Statgraphics software v.5.1 package (Manugistics, Rockwell MD). Data of GC–O maximum odour intensity (I_{max}) were submitted to Kruskal-Wallis one-way analysis of variance and medians were compared basing on box-and-whisker plot (Nuzzi et al. 2008). Data of $2^{-\Delta Ct}$ values, aroma amounts and GC–O peak area (A) were submitted to one-way ANOVA, and means were compared by Tukey's test at $p \leq 0.05$. Data of GC-O peak area were submitted to the principal component analysis (PCA) on the variance matrix.

RESULTS AND DISCUSSION

The SSC and TA values (Table 2) indicated good organoleptic properties of tested strawberries. They confirmed results obtained in previous three-year trial conducted in Italy on 14 strawberry cultivars for which the average SSC was 5.8-5.9 °Bx and the average TA was 8.5-9.7 meq·100 g^{-1} fw (Maltoni et al. 2002).

Table 2. SSC (°Bx) and TA (meq·100 g^{-1}) of strawberry fruit at full ripening. The means indicated by the same letter do not differ significantly according to Tukey HSD test at p = 0.05

	Genotypes			
	RdV	PdT	Onda	VR177
SSC	11.52 a	9.13 b	8.11 b	7.85 b
TA	21.04 a	17.03 b	12.28 c	12.82 c

The ester and terpene concentrations were significantly different among analysed genotypes (Table 3 & 4). The highest amount of methyl 2-methylbutanoate and ethyl hexanoate was detected in fruits of 'Profumata di Tortona'. The 'Profumata di Tortona' ester profile showed the prevalence of ethyl butanoate, the character impact compound responsible for "sweet" and "fruity, strawberry" descriptors. Ethyl butanoate was also the prevalent ester in selection VR 177. The *F. vesca* cultivar terpene profile was characterized by the prevalence of limonene. The α-terpineol was produced by all genotypes but its amount

was significantly higher in VR177. The sesquiterpene nerolidol was found only in the *F.* × *ananassa* genotypes and the higher concentration was noted for 'Onda'. The fruits of *F. moschata* produced more methyl 2-methylbutanoate and ethyl hexanoate than the other genotypes. The highest total concentration of esters was found in fruits of *F.* × *ananassa* genotypes.

The odorous events (OE) detected, with their retention indices RI (Kováts 1958), areas and intensities, are listed in Table 5. The GC-O profile of cultivated strawberries indicates the key role of esters in their global aroma. In both *F.* × *ananassa* genotypes are reported I_{max} = 2 or 3 for the "sweet" and the "strawberry, fruity" descriptors. 'Onda' fruits, together with "chemical", "herbaceous, fruity", and "herbaceous, spicy" has also the "chemical, mushroom" descriptor, that might indicate the presence of octanol. The 'Profumata di Tortona' strawberry profile shows the maximum I_{max} value for the descriptors "citrus, floral", "fruity, strawberry", "herbaceous, fruity", and "fruity, floral" together with "herbaceous, bug" and "chemical", due to the presence of aldehydes such as hexanal and (E)-2-hexenal. In 'Reine des Vallées' fruits the maximal values were reported for the descriptors "herbaceous", "fruity, strawberry", "herbaceous, fruity" and "fruity, floral", confirming the typical "wood, strawberry" flavour of *F. vesca*. The PCA of GC-O area data extracted 5 components, explaining the 88.01% of total variance. The biplot made with the first two components explains only the 58.68% of total variance. (Fig.1). PC2 for RdV were separated from PC2 of other cultivars and was associated to the "spicy", "nut", "baked" "sweet" and "caramel" notes, indicating the peculiar profile characterized by the presence of furaneols and terpene alcohols. VR177 showed positive values for both PC and was associated to the "sweet", "citrus", "floral" and "strawberry" descriptors. The PC1 divided VR177 fruits from the other strawberries. 'Onda' and 'Profumata di Tortona' fruits were associated to "sweet", "herbaceous", "floral", "spicy" and "herbaceous" and "bug" notes, indicating the importance of aldehydes in determining their aroma profile.

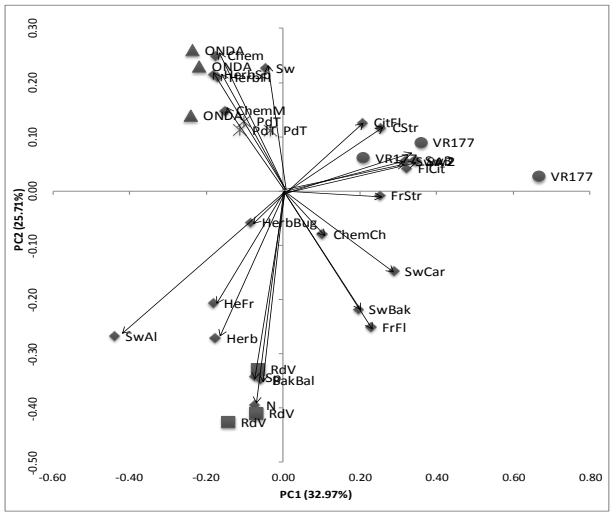

Fig. 1. GC-O data: principal component analysis of the areas of the odorous events. The odour codes are listed in Table 5; PdT = Profumata di Tortona; RdV = Reine des Vallées

Table 3. Concentrations (µg eq. hexyl acetate·100 g^{-1} f.w.) of esters identified in the SPME headspace. The means indicated by the same letter do not differ significantly according to Tukey HSD test at p = 0.05

Compound	Genotypes				
	P	RdV	PdT	Onda	VR177
Methyl butanoate	***	51.87 b	15.00 b	126.25 a	128.23 a
Methyl 2-methylbutanoate	***	0.0 b	19.51 a	1.22 b	3.62 a
Ethyl butanoate	ns	42.39	66.91	71.89	168.89
Butyl acetate	ns	2.60	7.91	5.51	9.88
Methyl hexanoate	ns	7.16	14.25	22.36	9.67
Ethyl hexanoate	***	0.95 bc	8.8 a	2.54 b	0.00 c
Hexyl acetate	ns	37.91	38.62	7.96	35.99

Table 4. Concentrations (µg eq. α–terpineol·100 g^{-1} f.w.) of terpenes identified in the SPME headspace. The means indicated by the same letter do not differ significantly according to Tukey HSD test at p = 0.05

Compound	Genotypes				
	P	*RdV	PdT	Onda	VR177
Limonene	***	784.28 a	108.12 b	36.01 b	0.00 b
Linalool	***	136.12 a	54.56 b	5.69 c	0.00 c
α–terpineol	**	71.27 b	110.19 b	141.48 ab	232.54 a
Nerolidol	***	0.0 b	0.0 b	223.27 a	70.62 b

Table 5. GC-O analysis. Odour descriptions: maximum odour intensities (medians, I_{max}) and GC-O peak areas (average, A) of the odorous events detected in the four genotypes

Odorous event		RI	A					I_{max}				
Code	Descriptor		*P*	RdV	PdT	Onda	VR177	*P*	RdV	PdT	Onda	VR177
SwAl	Sweet, alcohol	690	ns	785	0	0	0	**	1 a	0 b	0 b	0 b
HeFl	Herbaceous, Floral	815	***	0 b	0 b	317 a	0 b	**	0 b	0 b	1 a	0 b
SwAl2	Sweet, alcohol 2	853	**	0 b	0 b	0 b	838 a	**	0 b	0 b	0 b	1 a
Herb	Herbaceous	939	**	529 a	0 b	0 b	0 b	**	2 a	0 b	1 a	0 b
Sw	Sweet	983	*	0 b	0 b	302 a	1166 a	*	0 b	0 b	3 a	2 a
N	Nut	986	***	266 a	0 b	2699 a	0 b	**	1 a	0 b	0 b	0 b
CitFl	Citrus, Floral	1010	**	0 b	1590 a	0 b	1276 a	ns	0	2	0	2
FrStr	Fruity, strawberry	1040	ns	2078	1349	0 b	4320	ns	2	2	3	2
FlCit	Floral, Citrus	1059	ns	0	0	1791	3518	**	0 b	0 b	1 a	2 a
Chem	Chemical	1076	**	0 b	984 a	220	0 b	**	0 b	2 a	2 a	0 b
HerbBug	Herbaceous, Bug	1091	**	736 ab	1084 a	381 b	464 b	ns	1	2	1	1
HeFr	Herbaceous, Fruity	1228	ns	1549	1130	731	0	ns	2	2	2	0
ChemM	Chemical, mushroom	1300	*	0 b	0 b	926 a	0 b	**	0 b	0 b	2 a	0 b
CStrTerp	Strawberry, Terpene	1327	*	0 b	665 a	0 b	979 a	*	0 b	2 a	0 b	2 a
HerbSp	Herbaceous, spicy	1394	***	0 b	0 b	550 a	0 b	**	0 b	0 b	2 a	0 b
FrFl	Fruity, floral	1477	*	775 a	0 b	0 b	692 a	*	2 a	0 b	0 b	2 a
Sw2	Sweet 2	1525	***	0 b	0 b	0 b	1343 a	**	0 b	0 b	0 b	2 a
SwCar	Sweet, caramel	1605	*	492	0	0	801	**	1 a	0 b	0 b	2 a
SwBak	Sweet, baked	1700	***	620 a	0 b	0 b	720 a	*	1 a	0 b	0 b	1 a
ChemCh	Chemical, cheese	1744	ns	583	0	427	959	*	1 a	0 b	1 a	2 a
BakBal	Baked, balsamic	1800	*	665 a	0 b	0 b	0 b	**	1 a	0 b	0 b	0 b
Sp	Spicy	1873	ns	483 a	0 b	0 b	0 b	**	1 a	0 b	0 b	0 b
Sw3	Sweet 3	2250	***	0 b	0 b	0 b	783 a	**	0 b	0 b	0 b	2 a

I_{max}: The medians indicated by the same letter do not differ significantly at the 95% confidence interval (Box-and-Whiskers plot); A: The means indicated by the same letter do not differ significantly according to Tukey HSD test at p = 0.05

Table 6. $2^{-\Delta Ct}$ values of the tested genes. The means indicated by the same letter do not differ significantly according to Tukey HSD test at p = 0.05

Gene	Genotypes				
	P value	*RdV	PdT	Onda	VR177
SAAT	***	1.1×10^{-3} b	3.5×10^{-3} a	3×10^{-3} a	5.6×10^{-4} c
FaNES1	***	5.2×10^{-8} d	2.1×10^{-6} c	4×10^{-4} a	5×10^{-5} b
FvPINS	***	2.7×10^{-4} a	4.9×10^{-4} a	8.5×10^{-6} b	1.3×10^{-5} b
PINS1	***	1.1×10^{-4} a	1.6×10^{-4} a	5.7×10^{-6} b	6.9×10^{-6} b

P value: p-probability of the F statistic from ANOVA, *** $p \leq 0.001$

Parallel to biochemical study we analysed the expression of genes involved in catalysing the formation volatile ester in ripening fruits (*SAAT*) and responsible for terpenoid biosynthesis (*FaNES1*, *FvPINS* and *PINS1*). To date, only few genes that directly influence fruit flavour biogenesis have been reported in *Fragaria* genus. Genes coding alcohol acyltransferases, lipoxygenases and terpene synthases were identified in both, wild and cultivated species by Aharoni et al. (2000; 2004), meanwhile O-methyltransferase, eugenol synthase and quinone oxidoreductase genes were described for the cultivated strawberry by other authors (Raab et al. 2006; Zorrilla-Fontanesi et al. 2012). The *SAAT* gene, coding multifunctional acyltransferase plays a crucial role in aroma biochemistry (St- Pierre et al. 1998; Aharoni et al. 2000). Simultaneously, the biosynthesis of the terpenoids requires the action of monoterpene and sesquiterpene synthases. The study of Aharoni et al. (2000) revealed that *FaNES*1 gene showed high expression level in cultivated strawberry, but not in wild *Fragaria* species. *FvPINS* presented an inverse correlation and expressed only in the fruits of wild species (Aharoni et al. 2000). In our study the expression of flavour associated genes varied significantly among *Fragaria* genotypes (Table 6). The level of *SAAT*-gene expression was relatively high in fruits derived from 'Profumata di Tortona' and 'Onda' cultivars. Furthermore, the cultivated strawberry showed a more elevated induction of *FaNES1*, whereas the expression of *FvPINS* and *PINS1* genes was higher in fruits derived from wild then from cultivated species. These data support the results of Aharoni et al. (2000; 2004) and suggest how the differences in expression level of analysed genes reflect the metabolic diversity in flavour composition among fruits of cultivated and wild genotypes of *Fragaria*.

The molecular and biochemical analysis showed high correlations. According to them, the formation of volatile esters in ripe fruits was dictated by the expression of *SAAT* gene, especially for *F. moschata* and *F.* × *ananassa* strawberries. The sesquiterpene nerolidol was not found in fruits originated from wild species, characterized by high amounts of monoterpenes and showing an up-regulation of *FvPINS* and *PINS1* genes, involved in the formation of these compounds. α-Terpineol was found in high amount in fruits of VR 177, a product of the modern breeding oriented to the enhancement of the flavour characteristics.

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