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Identification of main fine or flavour components in two genotypes of the cocoa tree (*Theobroma cacao* L.)

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Summary

Cocoa seeds are the key raw material in chocolate manufacturing. Traders separate them into bulk and fine or flavour cocoa. The latter is characterized by the presence of special aroma notes (e.g. fruity). In contrast to chocolate aroma that derives from seed endogenous components (storage proteins, carbohydrates) fine aroma has been linked to the fruit pulp but, detailed information on its molecular background is lacking.

In the present study we analyzed fruit pulp and seeds of two fine or flavour cocoas (SCA6, EET62) and a bulk cocoa (CCN51) using GCMS.

The monoterpenes β-myrcene, β-*trans*-ocimene, β-*cis*-ocimene and β-linalool were characteristic for the SCA6 volatile composition. Regarding EET62 the secondary alcohol 2-heptanol, its ester 2-heptanol acetate and the methylketones 2-heptanone and 2-nonanone were typical. We conclude that these molecules are main components of SCA6 and EET62 fine aroma. Accordingly, such components may derive from different metabolic pathways depending on the genotype.

Introduction

The cocoa tree *Theobroma cacao* L. is native to the tropical rain forest of the eastern Andean slopes (Bartley, 2005). It is a major and stable base of agricultural income for millions of farmers in Africa, Asia, Central- and South America. Its seeds are the key raw material of the multi-billion-dollar chocolate industry (Motamayor et al., 2008). *T. cacao* is characterized by a huge genetic diversity (Bartley, 2005; Motamayor et al., 2008). Depending on the variety traders separate cocoa seeds into bulk cocoa and fine or flavour cocoa with the latter being characterized by the presence of special aroma notes such as floral and fruity absent in bulk cocoa (Ziegleder, 1990; Sukha et al., 2008). Fine cocoa is of higher value than bulk cocoa (International Trade Centre, 1991; Donovan, 2006).

Cocoa seeds consist of two large storage cotyledons and an embryo axis enclosed by a hardened but flexible seed shell. Attached to the seed shell elongated pectin rich endocarp cells form a fruit pulp (Duncan and Todd, 1972; Figueira et al., 1993; Andersson et al., 2006). The storage components in the cotyledons comprise 50 % fat, 15 % phenolics, 12 % protein, 5 % starch and 2 % sucrose on a dry weight base (Reineccius et al., 1972; Berbert, 1979; Schmieder and Keeney, 1980; Kim and Keeney, 1984; Clapperton, 1992; Voigt et al., 1993; Bucheli et al., 2001; Nazaruddin et al., 2001). The aqueous pulp contains 12 % mono- and disaccharides, 2 % citric acid as well as further organic acids, esters, aldehydes, methylketones, secondary alcohols and terpenes (Pettipher, 1986; Figueira et al., 1993; Quijano et al., 2010).

Fresh untreated cocoa seeds are characterized by an astringent taste due to the high content of phenolics especially anthocyanins (Jinap et al., 2005). They do not develop any chocolate aroma during chocolate manufacturing because they do not contain the necessary aroma precursors. The latter are developed during the postharvest treatment (e.g. Ziegleder and Biehl, 1988) that the seeds undergo in the countries of origin. This postharvest treatment consists of fermentation and drying. During fermentation the fruit pulp surrounding the cocoa seeds is degraded by yeasts, lactic- and acetic acid bacteria resulting in lactic and acetic acid formation as well as in a development of heat. The acids permeate into the seed tissue. This acidification and the heat cause seed death. Subsequently, the storage proteins and carbohydrates are degraded by the respective seed enzymes yielding peptides, free amino acids and reducing sugars: the chocolate aroma precursors (reviewed by Schwan and Wheals, 2004; Afoakwa et al., 2008). Thus, the chocolate aroma derives from seed endogenous components. Subsequent to fermentation the cocoa seeds are dried to a final moisture content of approximately 7 % in order to enhance storability. Drying and in particular fermentation also result in a notable reduction of astringency due to enzymatic oxidation of the phenolics (Kim and Keeney, 1984; Afoakwa et al., 2008). Fermented and dried cocoa seeds are named raw cocoa. The storage cotyledons of this product are used in the following process of chocolate manufacturing. This process comprises roasting of the cotyledon tissue. During roasting peptides, free amino acids and reducing sugars undergo Maillard reactions and Strecker degradation leading to chocolate aroma compound development (reviewed by Afoakwa et al., 2008). Numerous studies have been carried out on these compounds and dominant odour-active substances such as aldehydes (e.g. 3-methylbutanal), pyrazines (e.g. 2,3-diethyl-5-methylpyrazine) and furans (e.g. 2-methyl-3-(methyldithio)furan) have been identified in cocoa mass (Schnermann and Schieberle, 1997; Schieberle and Pfnuer, 1999; Counet et al., 2002; Taylor, 2002; Granvogl et al., 2006; Reineccius, 2006; reviewed by Afoakwa et al., 2008).

In contrast to the seed endogenous formation of the chocolate aroma precursors fine flavour notes have been linked to the fruit pulp (e.g. Eskes et al., 2007). These authors evaluated the sensory properties of the fruit pulp of several cocoa genotypes including EET62 (Estation Experimental Tropical Pichilingue clone 62) a fine cocoa with fruity and floral notes as well as CCN51 (Coleccion Castro Naranjal clone 51) a bulk cocoa lacking fine flavour. The sensory properties of the respective fruit pulps matched the descriptions given for raw cocoa of these genotypes leading Eskes et al. (2007) to propose that the corresponding components are pulp derived and already present when cocoa is harvested. Apparently, these components – like acetic acid – permeate into the seed tissue during fermentation and are retained throughout the drying process.

It has been repeatedly suggested that monoterpenes such as linalool are part of the components or rather molecules responsible for fine flavour in cocoa (e.g. Ziegleder, 1990). These authors report that fine or flavour cocoas contain higher amounts of linalool than bulk cocoa does (Ziegleder, 1990). Besides linalool, fermented and dried cocoa seeds also may contain the monoterpenes myrcene and ocimene (Ziegleder, 1990) described as spicy and floral, respec-

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tively (MOSCIANO, 1996 and 2000). Hence these molecules may be important for fine flavour as well. Linalool, myrcene and ocimen have been detected in the pulp of fresh ripe cacao fruits (QUIJANO et al., 2010) furnishing further evidence to the hypothesis of ESKES et al. (2007) that fine aroma components are pulp-derived. In total QUIJANO et al., (2010) could identify 66 different components in the pulp of an undefined cacao genotype typical for Columbian plantations among them methylketones such as 2-heptanone and 2-nonanone as well as secondary alcohols like 2-pentanol and 2-heptanol. Short-length methylketones (C_5-C_{11}) are highly potent flavour molecules (SCHWAB et al., 2008). Secondary alcohols are aroma active compounds as well with 2-pentanol and 2-heptanol being important components of passion fruit flavour (STROHALM et al., 2007; SCHWAB et al., 2008). Tab. 1 summarizes those components that in the analysis of QUIJANO et al. (2010) were contributing to 1 % or more of the total volatile amount. Interestingly, 2-pentanol and 2-heptanol have also been detected in roasted cocoa powder (BONVEHÍ, 2005). 2-heptanol also was found in dark chocolate (COUNET et al., 2002). Moreover, COUNET et al. (2004) report the presence of 2-heptanone and 2-nonanone in New Guinea cocoa liquor. Thus, besides monoterpenes also methylketones and secondary alcohols may play a key role in fine flavour formation. However, detailed information on the molecular background of cocoa fine flavour and its genotypic plasticity is lacking.

In the present study we determined the fruit pulp and unfermented seed volatile components of the cacao genotypes SCA6 (Scavina clone 6) – well known for its floral aroma notes in fruit pulp and raw cocoa (e.g. ESKES et al., 2007) – EET62 and CCN51 using gas chromatography-mass spectrometry (GCMS). Monoterpenes were characteristic for the volatile composition of SCA6 pulp and fresh seed tissue. In contrast, EET62 contained methylketones, secondary alcohols and their respective esters. We suggest that these molecules are a major source of fine flavour in SCA6 and EET62 and conclude that fine aroma components may derive from different metabolic pathways depending on the genotype.

Tab. 1: Volatile components in *T. cacao* pulp of an unknown genotype (from QUIJANO et al., 2010)
Only components corresponding to ≥1% of the total volatile amount are shown.

Number	Compound	Amount [%]
1	2-methyl-3-buten-2-ol	2.6
2	2-pentanol	1.4
3	2-pentyl acetate	25.0
4	2-heptanone	1.1
5	2-heptanol	1.6
6	2-heptyl acetate	29.7
7	*cis*-linalool oxide	3.4
8	2-nonanone	1.5
9	linalool	11.7
10	1-phenylethyl acetate	1.8
11	isoamyl benzoate	2.3

Materials and methods

Cocoa fruits

Cacao fruits of the genotypes SCA6 (Scavina clone 6), EET62 (Estation Experimental Tropical Pichilingue clone 62) and CCN51 (Coleccion Castro Naranjal clone 51) were provided by the International Cacao Collection at CATIE (Centro Agronómico Tropical de Investigación y Enseñanza), Turrialba, Costa Rica. All fruits were harvested during main crop in 2012. Material from three individual shipments in April, May and June was used in the experiments. Fruits with differing pulp appearance e.g. with a different degree of ripeness were discarded. A total of eight fruits were analyzed per genotype.

Volatile extractions

Fruit pulp and cotyledon tissue of freshly opened fruits were separated using a scalpel and the pulp was transferred immediately into 20 mL head space vials (Agilent Technologies Inc., Mississauga, Canada). The cotyledon tissue was broken into nibs and also transferred to head space vials. All vials were filled with an equal amount (~5 g) of tissue. Subsequently, the samples were equilibrated to a temperature of 35 °C in a water bath. The volatiles in the head space were collected by solid phase micro extraction (SPME) for 15 min using a 65 μm polydimethylsiloxane-divinylbenzene (PDMS/DVB) fiber (Supelco, Mississauga, Canada).

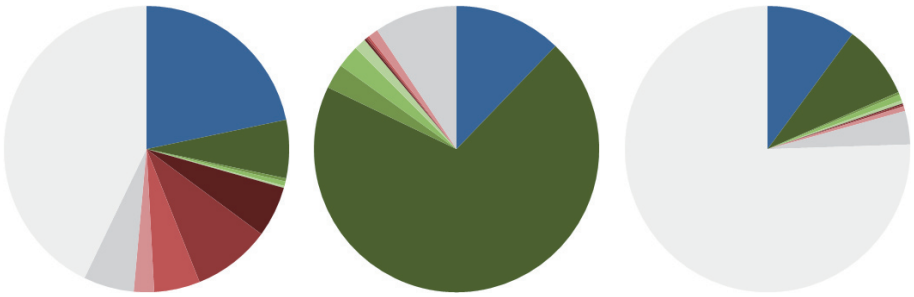
Volatile component analysis

The volatile analyses were performed on an Agilent 6890N Gas Chromatograph (Agilent Technologies Inc., Mississauga, Canada) and 5975 Inert XL Mass Spectrometer in an electron ionization (EI) mode at 70 eV. A J&W DB-WAX column (model number 122-7032, Agilent Technologies Inc., Mississauga, Canada) with an inner diameter of 250 μm, 0.25 μm film thickness and a length of 30 m was used. The GC temperature program was: 40 °C for 3 min, 3 °C*min^{-1} to 100 °C, 10 °C*min^{-1} to 150 °C, 15 °C*min^{-1} to 240°C, hold 5 min with the pulsed splitless injector temperature held at 250 °C. The pulsed pressure was set at 50 psi. The carrier gas (helium) flow was adjusted to 1 mL*min^{-1}. Samples were injected manually. The volatile components were identified by comparison of their mass spectra to the reference mass spectral data base of the National Institute of Standards and Technology MS library searches (Wiley W9N08). Moreover, the identification of substances referred to as major components of the volatile compositions of the three genotypes as shown in Fig. 1 and 3 was verified using authentic standards.
The quantity of volatile components was calculated based on the peak area and is given in percent. Total peak area observed with pulp from EET62 was used as reference area in order to facilitate comparison between the genotypes as well as between fruit pulp and cotyledon tissue. All volatile component amounts are referred to the fresh weight. Quantifications are based on the analyses of eight independent biological replicates (i.e. eight fruits) per clone. Three technical replicates were carried out per fruit.

Results

Pulp volatile components

Fruit pulp of clone EET62 had the highest overall amount of volatile components when compared to SCA6 and CCN51. The amount was more than 2-fold higher than in the former and four times higher in comparison to the latter (Fig. 1). The volatile composition of EET62 pulp was dominated by 2-heptanol acetate which contributed



Volatile composition	Genotype	SCA 6	EET 62	CCN 51
	Compound	**Amount [%]**	**Amount [%]**	**Amount [%]**
	2-pentanol acetate	21.70 ± 2.46	12.20 ± 2.05	10.14 ± 4.39
	2-heptanol acetate	6.57 ± 1.75	69.89 ± 19.72	8.34 ± 5.28
	2-heptanol	0.35 ± 0.11	2.82 ± 0.52	0.29 ± 0.14
	2-heptanone	0.52 ± 0.20	2.58 ± 0.77	0.69 ± 0.19
	2-nonanone	0.22 ± 0.07	1.35 ± 0.45	0.35 ± 0.10
	β-myrcene	5.73 ± 1.04	0.23 ± 0.20	0.14 ± 0.11
	β-*trans*-ocimene	8.84 ± 1.04	0.19 ± 0.14	0.19 ± 0.18
	β-*cis*-ocimene	5.21 ± 0.65	0.23 ± 0.21	t
	β-linalool	2.29 ± 0.61	1.12 ± 0.48	0.54 ± 0.33
	others	5.72	9.41	3.78
	dif. to reference	42.83	reference	75.52

Fig. 1: Cacao fruit pulp volatiles of genotypes SCA6, EET62 and CCN51
Volatiles of the fresh fruit pulp were collected by SPME and analyzed using GCMS. Quantities were calculated based on the peak area. The total peak area obtained with EET62 fruit pulp was used as reference area. All quantities are referred to the fresh weight (1g). Only compounds contributing to 1% or more of the total volatile amount are shown. Substances occurring in smaller quantities are grouped as "others". The number of fruits analyzed per genotype was eight (**n = 8**). Per fruit 3 replicates were carried out. Error values are given as standard deviation.
dif. to reference = difference to reference; the total peak area obtained for fruit pulp volatile components of EET62 is set as 100% (reference). In comparison to this reference area the total peak area obtained for SCA6 and CCN51 fruit pulp volatile components is smaller. The difference to reference is used to complete the diagrams of SCA6 and CCN51 to 100%; **t** = traces
Characteristic for the volatile composition of EET62 fruit pulp is the high amount of 2-heptanol acetate. Typical also are the notable quantities of 2-heptanol, 2-heptanone and 2-nonanone. In contrast, SCA6 contains high amounts of β-myrcene, β-*trans*-ocimene and β-*cis*-ocimene. 2-pentanol acetate was detected in relatively high quantities in all three genotypes. However, CCN51 contains far less volatiles than SCA6 and EET62 do.

to almost 70 % of the total volatile amount (Fig. 1). In SCA6 and CCN51 only 6.6 ± 1.8 and 8.3 ± 5.3 % of 2-heptanol acetate were measured, respectively. Moreover, EET62 was found to contain 2.8 ± 0.5 % heptanol, 2.6 ± 0.8 % heptanone and 1.4 ± 0.5 % nonanone. Regarding SCA6 and CCN51 these substances contributed to less than 0.7 % of the volatiles (Fig. 1).
In contrast to EET62, the volatile composition of SCA6 was characterized by high amounts of monoterpenes. The pulp contained 5.7 ± 1.0 % β-myrcene, 8.8 ± 1.0 % β-*trans*-ocimene and 5.2 ± 0.7 % β-*cis*-ocimene. In EET62 and CCN51 less than 0.3 % was measured regarding each of these substances. Moreover, 2.3 ± 0.6 % β-linalool was detected in SCA6. Considerable amounts of this monoterpene were also found in EET62. Its pulp contained 1.1 ± 0.5 %. In CCN51 only 0.5 ± 0.3 % β-linalool was measured (Fig. 1).
Pulp of all three clones contained notable amounts of 2-pentanol acetate. However, with 21.7 ± 2.5 % SCA6 contained approximately twice as much as EET62 and CCN51, respectively. Despite of the considerable amounts of 2-heptanol acetate and 2-pentanol acetate CCN51 was characterized by a low total content of volatile components in comparison to EET62 and SCA6 (Fig. 1).

Also regarding components contributing to less than 1 % of the total volatiles grouped in the partition "others" distinct differences were observed between SCA6 and EET62 on the one hand and CCN51 on the other. The former contained approximately 0.10 ± 0.06 % and 0.05 ± 0.03 % of α-farnesene as well as 0.08 ± 0.03 % and 0.09 ± 0.04 % acetophenone, respectively. No α-farnesene could be detected in the fruit pulp of CCN51. The amount of acetophenone did not exceed 0.01 %. Moreover, trace amounts of perillen and 2-undecanone were found in the samples from SCA6 and EET62. In CCN51 neither perillen nor 2-undecanone could be detected (Fig. 2).

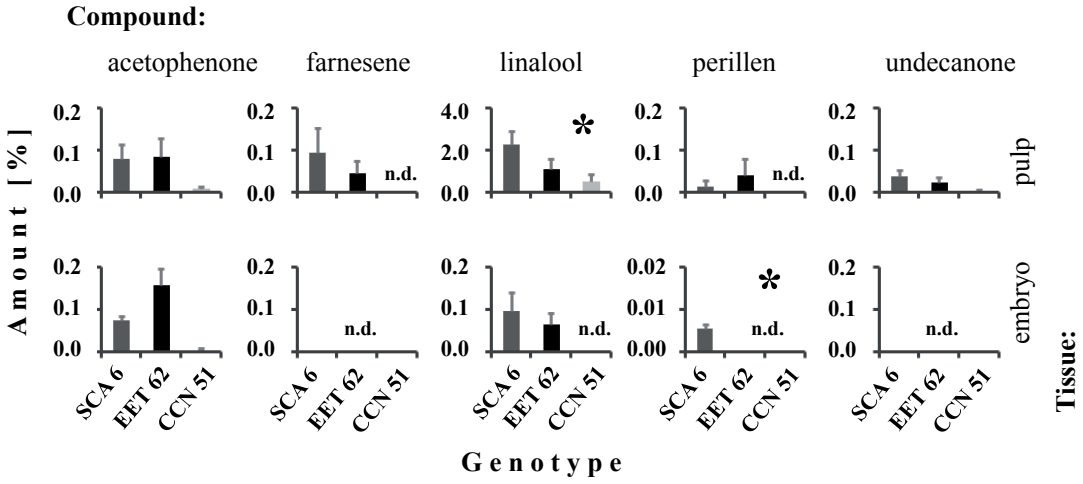


Fig. 2: Cacao fruit pulp and cotyledon tissue volatiles of genotypes SCA6, EET62 and CCN51 occurring in low amounts
Fresh fruit pulp as well as fresh untreated cotyledon tissue was transferred into head space vials and volatile components were collected by solid phase micro extraction. The volatile composition was analyzed using GCMS. Quantities were calculated based on the peak area. The total peak area obtained with EET62 fruit pulp was used as reference area. All quantities are referred to the fresh weight (1g). The number of fruits analyzed per genotype was eight (**n = 8**). Per fruit 3 replicates were carried out. Error values are given as standard deviation.
n.d. = not detectable
Apart from the β-linaool quantities in the fruit pulp where no distinct differences between EET62 and CCN51 could be observed all substances shown occurred in significantly higher amounts in EET62 and SCA6 than in CCN51. In some cases (e.g. α-farnesene) the respective components could be detected only in the fruit pulp and not in the cotyledon tissue.

Cotyledon volatile components

Like in the fruit pulp the highest overall amounts of volatile components in the cotyledon tissue were found in the EET62 samples. Their total volatile content was 1.6 fold higher than in SCA6 and 7.4 fold higher compared to CCN51 (Fig. 3). However, in contrast to the pulp the cotyledon volatile composition of EET62 was not dominated by 2-heptanol acetate which contributed to less than 2 % of the total volatiles. Remarkable were instead the 5.9 ± 1.7 % of 2-heptanol and the 6.6 ± 1.6 % of 2-heptanone. This corresponds to about twice the amount found in the fruit pulp and is about 40 times more than in SCA6 and CCN51 where less than 0.2 % of 2-heptanol and 2-heptanone were measured. Moreover, 0.7 ± 0.3 % 2-nonanone could be detected in the EET62 samples. In SCA6 and CCN51 only trace amounts (<0.1 %) were found. 2-heptanol acetate contributed to less than 0.1 % of the total volatiles in case of CCN51 and to only 0.12 ± 0.07 % regarding SCA6 (Fig. 3).

Characteristic for the volatile composition of SCA6 cotyledon tissue instead were the monoterpenes β-myrcene, β-*trans*-ocimene and β-*cis*-ocimene contributing to 2.0 ± 0.3 %, 2.4 ± 0.2 % and 0.8 ± 0.2 % of the total volatiles, respectively. In EET62 as well as CCN51 only trace amounts (<0.1 %) of these substances were found. β-myrcene could not be detected in CCN51 cotyledon tissue (Fig. 3). SCA6 cotyledon tissue also contained the highest amount of β-linalool. But, β-linalool contributed to only 0.10 ± 0.04 % of SCA6 total volatiles (Fig. 2). Characteristic was further the amount of 2-pentanol acetate. With 1.3 ± 0.5 % it was 5 times higher than in the EET62 and CCN51 samples, respectively (Fig. 3). Moreover, 2-pentanol that was only found in traces in the pulp contributed to 3.3 ± 0.7 % of the SCA6 cotyledon volatiles. However, also EET62 cotyledons contained 1.5 ± 0.5 % of this substance (Fig. 3).

2-pentanone was detected in considerable amounts in the tissue of all three genotypes. But, SCA6 and EET62 contained about twice the amount measured with CCN51. In general, CCN51 cotyledon tissue was characterized by a very low total amount of volatiles in comparison to SCA6 and EET62 (Fig. 3).

In the cotyledon tissue like in the pulp there are distinct differences between the three clones regarding several of the components occurring in very small amounts. Perillen for instance could only be detected in SCA6. However, it contributed to only 0.006 ± 0.001 % of the total volatiles. Acetophenone was found in the tissue of all three clones but, with 0.16 ± 0.04 % EET62 contained twice as much as SCA6. In CCN51 tissue less than 0.01 % was measured. α-farnesene as well as 2-undecanone could neither be detected in SCA6 and EET62 nor in CCN51.

Sensory characteristics of the pulp and cotyledon volatile components

Many of the components that we indentified in pulp and cotyledon tissue of EET62 and SCA6 are flavoring substances with frequent use in the food and perfume industry. Therefore, many of them have been evaluated individually for their sensory properties. 2-heptanol characteristic for the volatile composition of EET62 pulp and cotyledon tissue (Fig. 1 and 3) for instance is described as fruity, lemon grass and floral with regard to the odour. 2-nonanone is described as fresh and sweet regarding the odour and as fruity regarding the taste. Based on these descriptions 2-heptanol and 2-nonanone may be divided into the odour types citrus and fruity, respectively (Mosciano, 1991c; The Good Scents Company). Odour and taste of 2-heptanol acetate dominating the pulp volatile composition of EET62 and 2-heptanone present in high amounts especially in EET62 cotyledon tissue are described as fruity (Mosciano, 1991b; The Good Scents Company).

In contrast, the monoterpene β-*cis*-ocimene typical especially for the pulp but, also for the cotyledon tissue of SCA6 is described as floral regarding the odour. β-linalool characteristic for the pulp (Fig. 1 and 3) is as well classified floral with regard to the odour. Moreover, also the taste of β-linalool is defined floral. β-*cis*-ocimene and β-linalool are of the same odour type: floral (Mosciano, 1996; The Good Scents Company). The sensory properties of β-myrcene and

Volatile composition			
Genotype	**SCA 6**	**EET 62**	**CCN 51**
Compound	**Amount [%]**	**Amount [%]**	**Amount [%]**
2-pentanol acetate	1.32 ± 0.48	0.29 ± 0.09	0.25 ± 0.23
2-pentanol	3.25 ± 0.74	1.49 ± 0.48	0.65 ± 0.17
2-pentanone	1.62 ± 0.57	1.85 ± 0.58	0.90 ± 0.46
2-heptanol acetate	0.12 ± 0.07	1.45 ± 0.63	t
2-heptanol	0.16 ± 0.05	5.87 ± 1.70	0.12 ± 0.10
2-heptanone	0.16 ± 0.02	6.63 ± 1.59	0.16 ± 0.12
2-nonanone	t	0.71 ± 0.34	t
β-myrcene	1.96 ± 0.26	t	n.d.
β-*trans*-ocimene	2.37 ± 0.22	t	t
β-*cis*-ocimene	0.75 ± 0.15	t	t
others	0.53	1.22	0.46
dif. to reference	87.74	80.43	97.36

Fig. 3: Cacao cotyledon volatiles of genotypes SCA6, EET62 and CCN51
Fresh untreated cotyledon tissue was broken into nibs and volatiles were collected by SPME. Subsequently, volatiles were analyzed using GCMS. Quantities were calculated based on the peak area. The total peak area obtained with EET62 fruit pulp was used as reference area. All quantities are referred to the fresh weight (1g). Only compounds contributing ≥ 0.7% of the total volatile amount are shown. Substances occurring in smaller quantities are grouped as "others". The number of fruits analyzed per genotype was eight (**n = 8**). Per fruit 3 replicates were carried out. Error values are given as standard deviation.
dif. to reference = difference to reference; the total peak area obtained for fruit pulp volatile components of EET62 is set as 100% (reference). In comparison to this reference area the total peak area obtained for EET62, SCA6 and CCN51 cotyledon tissue volatiles is smaller. The difference to reference is used to complete the diagrams of EET62, SCA6 and CCN51 to 100%; **n.d.** = not detectable; **t** = traces
Characteristic for the volatile composition of EET62 cotyledon tissue are 2-heptanol, 2-heptanol acetate, 2-heptanone and 2-nonanone. Also 2-pentanol occurs in notable amounts but, it is found in higher quantities in SCA6. Characteristic for the latter are high amounts of β-myrcene, β-*trans*-ocimene and β-*cis*-ocimene. Also 2-pentanol acetate and 2-pentanol are typical for the SCA6 volatile composition. In contrast, CCN51 contains far less volatiles than SCA6 and EET62 do.

β-*trans*-ocimene typical for SCA6 pulp and cotyledon tissue as well are described as citrus and sweet, respectively (Mosciano, 2000; The Good Scents Company).
2-pentanol acetate detected in considerable amounts in the pulp of all three clones whilst in the cotyledons notable amounts were only typical for SCA6 is described as tropical and orange with regard to its sensory properties (Mosciano, 2009; The Good Scents Company). Odour and taste of 2-pentanol only contributing to high amounts of the volatiles in EET62 and especially SCA6 cotyledon tissue (Fig. 1 and 3) are described as fermented and alcoholic. The substance is classified into the odour type fermented (Mosciano, 2009; The Good Scents Company). 2-pentanone detected in considerable amounts in the cotyledon tissue of all three clones is described as fruity, banana-like and sweet (Mosciano, 1995). Tab. 2 provides a detailed description of the sensory properties of all fruit pulp and cotyledon tissue components reported.

Discussion

Potential fine aroma components of SCA6 and EET62

The individual sensory descriptions given for 2-heptanol, 2-heptanol acetate, 2-heptanone and 2-nonanone (Tab. 2) match the fine aroma characteristics of EET62 reported by Eskes et al. (2007). All four compounds were found to be present already in the fresh cotyledon tissue. Moreover, CCN51 having no fine flavour contained significantly lower amounts especially regarding the cotyledons (Fig. 1 and 3). Therefore, we conclude that 2-heptanol, 2-heptanol acetate, 2-heptanone and 2-nonanone are a major source of fine flavour in EET62. Interestingly, all four components have also been found in cocoa liquor (Counet et al., 2004). Influence on the fruity aroma notes in EET62 also may have 2-pentanone due to the relatively high content and moreover because its odour strength is rated "high" (The Good Scents Company). However, it is also present in notable amounts in CCN51 and hence may be of only marginal importance. 2-pentanol does not match EET62 sensory properties but might be

Tab. 2: Sensory properties of *T. cacao* pulp and cotyledon volatiles from genotypes SCA6 and EET62
The respective substances were evaluated individually. Sensory descriptions are given according to MOSCIANO (1991abc; 1995; 1996; 2000; 2009) and THE GOOD SCENTS COMPANY. Odour descriptions are shown in italics when the undiluted substance was tested (otherwise substance amount = 1-10%). Descriptions cited in the text are underlined.

Compound	Odour type	Odour description	Taste description
2-heptanol acetate	brown	fruity, fenugreek	fruity, fatty, green
2-heptanol	citrus	*fruity*, *lemon grass*, *floral*, *fresh*, *sweet*, *herbal*, *green*	-
2-heptanone	cheesy	fruity, sweet, coconut, spicy, herbal, woody	fruity, coconut, cheesy, waxy, green
2-nonanone	fruity	*fresh*, *sweet*, *green*, *weedy*, *earthy*, *herbal*	fruity, green, cheesy, dairy, buttery
β-myrcene	spicy	*balsam*, *peppery*, *terpene*, *spicy*	citrus, fruity, woody, vegetative
β-*trans*-ocimene	herbal	*sweet*, *herbal*	-
β-*cis*-ocimene	floral	*floral*, *flower*, *sweet*, *warm*, *herb*	-
β-linalool	floral	*floral*, *citrus*, *sweet*, *blueberry*, *woody*, *green*	floral, citrus, orange, lemon, waxy, woody
2-pentanol acetate	herbal	*tropical*, *herbal*,	tropical, orange
2-pentanol	fermented	*fermented, fusel, mild*, *green*	alcoholic, ripe banana, fusel, musty
2-pentanone	fruity	fruity, banana, sweet, wine, ethereal, woody	fruity, banana-like, sweet
acetophenone	floral	*sweet*, *almond*, *acacia*, *mimosa*, *hawthorn*, *pungent*	bitter almond cherry pit-like, powdery
perillen	woody	*woody*	-
α-farnesene	woody	*citrus*, *herbal*, *lavender*, *bergamot*, *neroli*, *myrrh*, *green*	fresh, green, vegetative
2-undecanone	fruity	*fruity*, *floral*, *waxy*, *creamy*, *fatty*	fruity, waxy, ketonic

perceived differently in the presence of the other volatile components. 2-undecanone described as fruity by MOSCIANO (1991a) may as well play role in the EET62 flavour composition. But, it was only present in trace amounts in the pulp and could not be detected in the fresh cotyledons underlining the potential importance of particularly 2-heptanol acetate, 2-heptanone and 2-nonanone for the fruity aroma notes of EET62. Of importance especially regarding the floral aroma notes may further be β-linalool, β-*cis*-ocimene and acetophenone. However, aside from the β-linalool content of the pulp all substances are present in very small amounts and hence might be below the threshold level for sensory perception.

The individual sensory description given for β-*cis*-ocimene and β-linalool (Tab. 2) match the fine aroma characteristics of SCA6 (ESKES et al., 2007). With their citrus and sweet aroma notes β-myrcene and β-*trans*-ocimene apparently also contribute to the floral character of this genotype. Together the four monoterpenes account for almost 50 % of the volatile compositions in pulp and cotyledon tissue (Fig. 1 and 3) underlining their potential importance for SCA6 fine aroma. Moreover, CCN51 only contains trace amounts of these compounds. Therefore, we suggest that β-myrcene, β-*trans*-ocimene, β-*cis*-ocimene and β-linalool – interestingly all components except β-*trans*-ocimene have also been found in raw cocoas (ZIEGLEDER, 1990) – are main molecules for SCA6 fine aroma. Hence, the potential key components of SCA6 fine aroma are monoterpenes. Their biosynthesis takes place via the methylerythritol 4-phosphate (MEP) pathway (RODRIGUEZ-CONCEPCIÓN and BORONAT, 2002; SCHWAB et al., 2008). In contrast, the potential key components of EET62 fine aroma consist of methylketones, secondary alcohols and their respective esters. These substances derive from the fatty acid metabolism (FRIDMAN et al., 2005; SCHWAB et al., 2008). Therefore, we conclude that depending on the genotype fine aroma components derive from different metabolic pathways.

Besides the above mentioned monoterpenes acetophenone may further enhance the floral character of SCA6. But, like discussed for EET62 the concentration of acetophenone might be below the sensory threshold level. Due to its tropical and orange character as well as its abundance in the samples it seems likely that 2-pentanol acetate contributes to the SCA6 dry fruit aroma note mentioned by ESKES et al. (2007). 2-pentanone classified into the odour type fruity might contribute to this aroma note as well. However, in CCN51 both substances are present in comparable amounts (Fig. 1 and 3). Thus, they may have only marginal influence on the overall fine aroma of SCA6. The high amount of 2-pentanol in the cotyledon tissue of SCA6 suggests that it also plays role in the aroma composition. Odour and taste are described as fermented and alcoholic but also as ripe banana (Tab. 2). Hence, it might be that 2-pentanol is responsible for the dry fruit character of SCA6 as well. However, during fermentation it may also react with the acetic acid migrating into the cotyledon tissue resulting in the formation of additional 2-pentanol acetate. To further investigate the role of the single components in EET62 and SCA6 fine flavour aroma extract dilution and aroma reconstitution trials as described for instance by HINTERHOLZER and SCHIEBERLE (1998) and BUETTNER and SCHIEBERLE (2001) may be carried out. Generally, it has to be considered that when using SPME the relative amount measured for a specific substance may vary depending on fibre affinity and competition with other volatiles for the fibre binding sites. However, the aim of our studies was not a quantification of the volatiles but the genotype specific comparison. In future experimental approaches precise quantification may be achieved using isotope dilution assays as described for instance by BUETTNER and SCHIEBERLE (2000).

In contrast to the cocoa volatile extraction method described here the method reported by QUIJANO et al. (2010) comprises blending of the fruit pulp using a juicer potentially resulting in enhanced compound oxidation. Volatiles were extracted from the headspace

above the supernatant of pulp homogenate (QUIJANO et al., 2010). To avoid enhanced volatile oxidation we did not apply blending. In total, QUIJANO et al. (2010) identified 66 volatile compounds from cacao pulp. Eleven of them correspond to 1 % or more of the total volatile amount and are listed in Tab. 1. 2-heptanol acetate and 2-pentanol acetate together contribute to more than 50 % of the total volatiles. Both substances also were detected in high amounts in our analyses (Fig. 1). Moreover, components occurring in relatively low quantities such as 2-pentanol, 2-heptanol, 2-heptanone and 2-nonanone (Tab. 1) could be detected following our extraction protocol (Fig. 1). β-myrcene, β-*trans*-ocimene and β-*cis*-ocimene contributing to less than 1 % of the total volatiles in the analyses of QUIJANO et al. (2010) were found in quantities exceeding 5 % (Fig. 1). In contrast, 1-phenylethyl acetate as well as isoamyl benzoate was detected in higher quantities by QUIJANO et al. (2010). In our samples these substances contributed to less than 1 % of the volatiles and therefore are not shown in Fig. 1. The reproducibility regarding samples taken from the same fruit (technical replicates) was very high. Thus, cocoa fruit pulp volatiles may also be extracted without previous pulp juicing. That *cis*-linalool oxide (Tab. 1) could not be detected in our analyses may be due to the avoidance of pulp juicing probably enhancing the formation of oxidation products. Also 2-methyl-3-buten-2-ol could not be detected in our samples. This might be explained by genotype specific variation also observed between SCA6, EET62 and CCN51 (Fig. 1).

Fine aroma component migration during fermentation

Cocoa seeds fermented in the presence of aromatic *Annona muricata* pulp revealed flavour attributes of this fruit after completion of the postharvest treatment (ESKES et al., INGENIC newsletter, accessed 20.12.12) proving that aroma components may permeate into the seed tissue during fermentation and that they may be retained during the drying process. The same can be expected regarding the potential SCA6 and EET62 pulp fine aroma components that we reported here. However, our findings also show that these substances are already present in the unfermented seeds and that some of them even occur in higher amounts in the fresh cotyledons than in the pulp. Moreover, preliminary results from fermentation-like incubations of SCA6 and EET62 seeds suggest that there is no increase in the potential fine aroma component content in the seed tissue (KADOW et al., unpublished data). Thus, fruity and floral flavour attributes may already be fully developed in the fresh seeds. However, this does not mean that fine flavour components are not pulp derived. Provided that the corresponding substances – in contrast to acetic acid that can not permeate the testa of fresh seeds (ANDERSON et al., 2006) – are able to cross the seed shell these can migrate into the cotyledon tissue already upon fruit ripening. Indeed, most of the potential fine flavour components such as 2-heptanol and β-*cis*-ocimene are insoluble or fairly soluble in water. Thus, a migration with the water stream upon initiating germination as suggested for acetic acid (ANDERSON et al., 2006) is rather unlikely. Once permeated through the testa such compounds may be retained in the fat phase of the cotyledons resulting in enrichment. Interestingly, with increasing temperatures as occurring during fermentation this retention of volatile components in a fat phase may be enhanced drastically (MAIER and KESSLER, 1977). Moreover, fat solubility would explain the retention of the corresponding substances during the drying process.

Fermentation-like incubations are suitable to reassemble chocolate aroma precursor formation in cocoa seeds at lab scale (BIEHL and PASSERN, 1982). However, it may be inappropriate for studying fine flavour component migration. Whether the potential fine aroma components permeate through the cocoa seed testa and if a migration from the pulp to the cotyledons takes place during fruit ripening and fermentation will be in the focus of our future research.

Quality controls

Whether raw cocoa is recognized as fine flavour cocoa by traders mainly is provenance dependent since *T. cacao* genotypes with fine flavour attributes derive from the selections carried out by Indians from several regions in Central- and South America (e.g. BARTLEY, 2005). Generally, fine or flavour cocoa is produced from Criollo or Trinitario cocoa-tree varieties, while bulk cocoa comes from Forastero trees (ICCO, 2011). However, with the continuous renewal of plantations and with the recent intensification of the breeding work on cocoa tree especially regarding yield and resistance major changes in the genetic structure of cocoa plantations have been taking place (AIKPOKPODION et al., 2006). This necessarily also has influence on the sensory attributes of the raw cocoa such as fine flavour. New genotypes may have a different fine aroma or even none. Moreover, fine cocoa requires shorter fermentation time than bulk cocoa does (AFOAKWA et al., 2008). Inappropriate fermentation may result in loss of previously present fine aroma. Thus, provenience and the cacao variety planted alone cannot guarantee fine flavour quality. The genotype-specific information on the identity of potential fine flavour components reported here allows the establishment of methods for a quick qualitative and quantitative evaluation of fine flavour properties of a given raw cocoa. Moreover, such evaluations could be used for fine flavour focused optimization of the postharvest treatment as well as the chocolate manufacturing processes. Work on such methods is underway.

Selections and breeding

Recent breeding projects on cocoa tree such as the USDA-ARS project initiated in 1999 (e.g. SCHNELL et al., 2007) are focusing on resistance and yield. Indeed, there are many reports on molecular marker development for instance regarding black and frosty pod as well as witches broom resistance or tolerance (QUEIROZ et al., 2003; RISTERUCCI et al., 2003; FALEIRO et al., 2006; BROWN et al., 2007; LIMA et al., 2008; LANAUD et al., 2009). Also regarding yield and butter fat content molecular markers have been described (SCHNELL et al., 2005; ARAUJO et al., 2009; MARCANO et al., 2009). In contrast, a development of molecular markers for genotype-specific fine aroma qualities has not been reported so far. Detailed information on the molecular identity of fine aroma components will be of great help in the development of such markers. The availability of molecular markers for fruity or floral flavour attributes would significantly alleviate and accelerate breeding work aiming at the integration of such attributes into trees with favorable traits regarding resistance and yield. This would not only guarantee the maintenance of specific fine flavour qualities but could also help to increase the income of cocoa farmers since fine cocoa can achieve prices twice as high as bulk cocoa (DONOVAN, 2006).

The methylerythritol 4-phosphate (MEP) pathway that monoterpenes and thus the potential key components of SCA6 fine aroma derive from is localized in the plastids (RODRIGUEZ-CONCEPCIÓN and BORONAT, 2002; SCHWAB et al., 2008). Methylketones apparently are synthesized by hydrolysis and subsequent decarboxylation of β-ketoacyl-ACPs (acyl carrier protein) by methylketone synthase (FRIDMAN et al., 2005; SCHWAB et al., 2008). In tomato the enzyme is localized in the stroma of the plastids (FRIDMAN et al., 2005). Methylketones are supposed to be the precursors of secondary alcohols such as 2-heptanol (STROHALM et al., 2007; SCHWAB et al., 2008). Thus, the different metabolic pathways that the potential fine aroma components of EET62 and SCA6 derive from apparently are located in the same cell compartments: the plastids. While the pathways are active in plastids, the genes encoding cocoa tree linalool synthase are localized in the nucleus on chromosome 6 (ARGOUT et al., 2011), and in general, all plant monoterpene synthases appear to be encoded in the nuclear genome

(CHEN et al., 2011). Nevertheless, plastids may have an impact on the regulation of gene expression in the nucleus via retrograde signaling (e.g. ECKARDT, 2011). Therefore, maternal inheritance of plastids (SVAB and MALIGA, 2007) should be taken into consideration when selecting paternal and maternal parents for breeding of fine aroma in cocoa.

Conclusions

Our study shows that cacao fruit pulp may have great quantitative and qualitative differences in the composition of volatiles depending on the genotype. Among these volatiles i.e. the potential fine aroma components are monoterpenes (typical for SCA6) as well as methylketones, secondary alcohols and their respective esters (typical for EET62). Consequently, fine aroma components apparently derive from at least two different metabolic pathways. The migration of these fine aroma components from the fruit pulp to the cotyledon tissue seems to take place already during fruit ripening whereas the typical basic chocolate flavour is composed upon fermentation.

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