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Diversity of metabolite patterns and sensory characters in wild and cultivated strawberries[1]

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Abstract. In breeding programs wild strawberry species are used increasingly to enhance the genetic diversity and to implement higher sensory quality. Next to *Fragaria moschata* L. and *F. viridis* Weston wild collections cultivars of *Fragaria vesca* L. were the standards for strawberry taste in Europe before the development of *Fragaria* × *ananassa* Duch. around 250 years ago. Therefore, the objective of this research was the evaluation of patterns of volatile organic compounds including around twenty strawberry character impact compounds evaluated for *Fragaria vesca* and in comparison for standard cultivars of *Fragaria* × *ananassa*. The metabolic patterns were measured using immersion-SBSE-GC-MS. Sixteen accessions of *Fragaria vesca* exhibit a high diversity in qualities and quantities of volatile organic compounds (VOCs). A specific fruit sampling strategy equalizes environmental caused variation and avoids misleading interpretations. The shown results are valuable for further breeding activities regarding flavor, transcriptomic analyses, studies of biochemical pathways and signaling compounds as well as marker development.

Keywords: Fragaria, aroma compounds, flavour, stir bar sorptive extraction, bio-diversity

1. Introduction

In general, wild strawberries have a higher aroma intensity compared with cultivated ones. The flavor quality differs significantly [16]. Before *Fragaria* × *ananassa* was developed by the hybridisation of *Fragaria chiloensis* (L.) Miller and *Fragaria virginiana* Miller around 250 years ago, cultivars of *Fragaria vesca*, *Fragaria moschata* and selections of *Fragaria viridis* existed in Europe. They cause sensory impressions which normally are not associated with cultivated strawberries. Cultivars of *F. vesca* and *F. moschata* are still grown in house gardens and as gourmet fruits in several regions in Europe. The wood strawberry *Fragaria vesca* L. is the most widely distributed species of the genus *Fragaria* and appears circumpolar throughout Europe, northern America, and northern Asia. Its impressive diversity with four subspecies and different forms is believed as an adaptation to the different requirements of the ecosystems which were occupied by this strawberry species. Cultivars of *F. vesca* were developed using at first mutations (forma *semperflorens*) and selections of large fruiting genotypes. In sensory evaluation, the knowledge about these cultivars serves as a 'background standard' for the evaluation of *F.* × *ananassa* cultivars. On the contrary, the notes of *F. vesca* are very intense and result sometimes even in negative sensory characteristics which are described as soapy, perfume-like or astringent [16].

In Europe, after the 1750's the cultivated wild-types were replaced by the garden strawberry (*F.* × *ananassa* Duch.) which brought bigger fruits and a unique pleasant flavor. Later, since the beginning of the 20th century *F. vesca* was

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used in breeding programs with *F.* × *ananassa* as donor for interesting sensory and resistance traits [2, 10, 13]. The development of synthetic octoploids [4, 7] was also based on *F. vesca* accessions.

F. vesca (2n = 2x = 14) with its small genome of 240 Mb and the possible day-neutral behavior (forma *semperflorens*) became the model plant for molecular studies in the Rose family (*Rosaceae*) [8]. Meanwhile, the genome sequencing was completed for a Hawaiian accession with white berries [14].

In this study, the diversity of volatile patterns in 16 *F. vesca* accessions was investigated in comparison to selected *F.* × *ananassa* cultivars using a representative sampling strategy. The results of this diversity study regarding volatile organic compounds (VOCs) are a basis for further breeding activities regarding flavor, transcriptomic analyses, studies of biochemical pathways and signalling compounds as well as marker development.

2. Material and methods

2.1. Plant material

Sixteen *F. vesca* accessions were cultivated in 2011 on the field at Dresden-Weixdorf, Germany, each in a quadratic area of 1 m² on sandy loam on gravel ground according to their natural habitats. Five cultivars of *F.* × *ananassa* were planted on the same field in rows. Fruits of one year old plants were harvested in 2011.

Fully ripe, healthy and typical fruits of 1 m² area of each *F. vesca* accession and all available typical healthy fruits from six clone plants of each *F.* × *ananassa* cultivar were harvested. Additionally, for the accession *F. vesca* ssp. *vesca* 'Korsika' (korsik) altogether five separated harvests were collected and analyzed to examine the influence of date of harvest on the volatile patterns. All fruits were immediately frozen at minus 20°C after the sepals had been removed. The following accessions were harvested: 1 – *F. vesca* f. *alba* St.08/101 (foralb); 2 – *F. vesca* f. *alba* 'South Queen Ferry' (sferry); 3 – *F. vesca* ssp. *vesca* St. 94, 13 'Baikal' (baikal); 4 – *F. vesca* ssp. *bracteata* St. 98, 04-4 (bracte); 5 – *F. vesca* ssp. *americana* St. 14324 (americ); 6 – *F. vesca* f. *semperflorens* 'Red Wonder' (redwon); 7 – *F. vesca* f. *semperflorens* 'Yellow Wonder' (yelwon); 8 – *F. vesca* ssp. *vesca* 'Island' (island); 9 – F. *vesca* ssp. *vesca* 'Kaiserpfalz Tilleda' (tilled); 10 – *F. vesca* ssp. *vesca* 'Korsika' (korsik); 11 – *F. vesca* ssp. *vesca* 'Multiplex' (multip); 12 – *F. vesca* ssp. *vesca* 'Weimar' (weimar); 13 – *F. vesca* ssp. *vesca* 'Böhmen (boehme); 14 – *F. vesca* ssp. *vesca* 'Tüchersfeld' (tuefel); 15 – F. vesca ssp. vesca 'Süd-Öland 1' (oeland); 16 – *F. vesca* ssp. *vesca* 'Großolbersdorf' (olbers); 17 – *F.* × *ananassa* cv. 'Alba' (ALBA); 18 - *F.* × *ananassa* cv. 'Mara de Bois' (MDB); 19 – *F.* × *ananassa* cv. 'Mieze Schindler' (MS); 20 – *F.* × *ananassa* cv. 'Polka' (POLKA); 21 – *F.* × *ananassa* cv. 'Elegance' (ELEG). The origin of these accessions is described in Ulrich and Olbricht [17].

2.2. Extraction of fruit volatiles and gas chromatography – mass spectrometry

An enzyme inhibited strawberry juice was produced using all frozen fruits of each accession or cultivar of the whole season in a mixture. One mass part of fruit were homogenized in 1.12 mass part of an aqueous solution of 18.6% (m/v) NaCl by a household mixer for 2 min followed by the centrifugation of the homogenate with 4000 rpm for 30 min. The supernatant (100 ml) were mixed with 10 µl internal standard (0.1% (v/v) 2,6-dimethyl-5-hepten-2-ol dissolved in ethanol). For each sample, three head-space vials containing 3 g NaCl each for saturation were filled with 10 ml of the supernatant, sealed with magnetic crimp caps including a septum. The vials were stored at 4°C until analysis (three weeks).

Fruit volatiles were isolated by immersion stir bar sorptive extraction (imm-SBSE) using the Twister™ device from Gerstel, Germany. Details of the extraction method and subsequent gas chromatography (GC) including a quadrupol mass spectrometric (MS) detector are described in Ulrich and Olbricht [17].

2.3. Data processing

For a non-targeted data processing, the chromatogram integration results were imported subsequently as raw data (txt-formatted) into the chemometrical software ChromStatTM 2.6 from Analyt-MTC (Müllheim, Germany). The

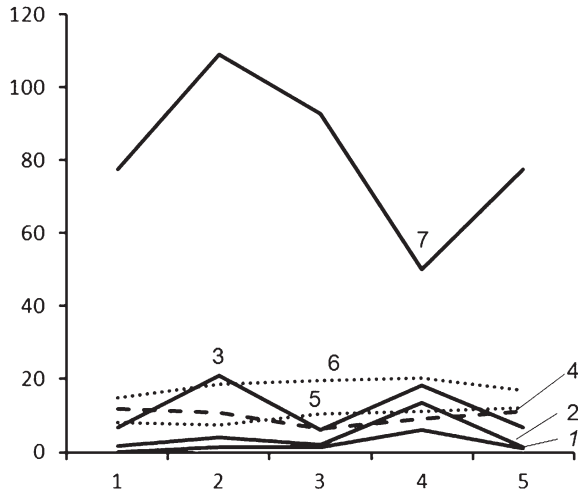

Fig. 1. Development of VOC concentrations of *F. vesca* ssp. *vesca* 'Korsika' over five harvest dates in one season. Y-axis: relative concentration in counts (peak area); x-axis number of harvest. Nomenclature of volatiles: 1: (*Z*)-3-hexenyl acetate; 2: hexyl acetate; 3: ethyl butanoate; 4: α-terpineol; 5: (*E*)-2-hexenol; 6 : 1-hexanol; 7: methyl anthranilate.

semi-quantitative results were expressed as non-dimensional values (counts or relative concentrations). Statistical analyses were performed using the software Statistica 7.1 from StatSoft (Tulsa, USA) and Multi Experiment Viewer from TM4 Software Development Team (www.tm4.org).

3. Results and discussion

The combination of imm-SBSE and GC-MS result in very detailed chromatograms with up to 200 distinct peaks of VOCs. To prevent overlooking of 'new' peaks a methodology using a non-targeted analysis data processing was used in the present research. This kind of analytics is an adequate approach to study biodiversity of accessions which were not analyzed before. Using only pre-built peak tables, unexpected substances might have been skipped in the data processing and might have caused a loss of information.

3.1. Dynamic of volatile patterns during the harvest season

In Fig. 1, the development of certain VOCs is demonstrated over five harvest dates of *F. vesca* ssp. *vesca* 'Korsika' (korsik, no. 10). The highest difference in concentration between the harvest dates was observed for the key compound methyl anthranilate (7) with its intense flowery aromatic note typical for wood strawberry and acacia. The values vary by a factor of two between values of 49.99 (harvest 4) and 108.94 (harvest 2). Further, the aroma-active esters (*Z*)-3-hexenyl acetate (1), hexyl acetate (2), ethyl butanoate (3) with typical fresh-fruity aroma notes are not stable between different harvest dates. In contrast, the concentration trend of the terpenoid α-terpineol and the alcohols (*E*)-2-hexenol and 1-hexanol (green, grassy odor) seem to be more stable over the development of harvest. The volatile pattern of the 2nd harvest date follows the rank 1, 2, 5, 4, 6, 3 and 7 in difference to the 4th harvest date with the rank 1, 4, 5, 2, 3, 6 and 7. The variation of volatile concentrations considerably influences the evaluation of aroma patterns. Consequently, the use of fruit samples from only one or a few harvest dates gives results which represent merely a 'snapshot' of the possible development of metabolite patterns and may result in misinterpretation of metabolic relationships. Therefore, the sampling strategy is important for the assessment of the diversity as depicted in Section 3.2. With the applied method a batch of all typical berries from the whole harvest season is covered.

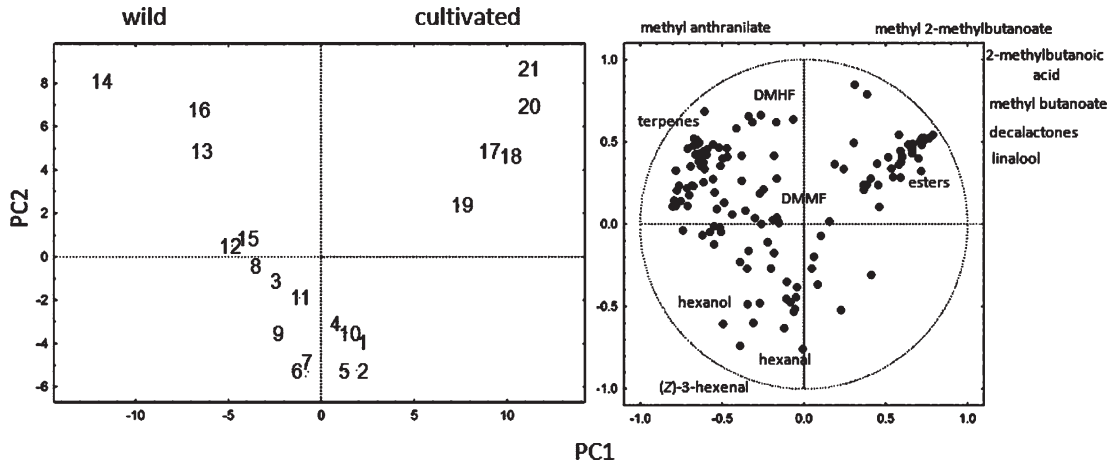

Fig. 2. Principal component analysis (PCA) using a data set of 16 *F. vesca* accessions and five *F.* × *ananassa* cultivars. The PCA was performed with altogether 194 peak signals. A number of 67 compounds out of the 194 peaks were identified by MS library search (tentatively identified) and, if possible, by co-elution of authentic substances (fully identified).

3.2. Diversity of volatile patterns in one harvest season

The whole data set of 16 *F. vesca* accessions and five diverse cultivars consisting of 194 peaks each comprises 4074 metabolic data. The total amount of volatile metabolites calculated by summation of all 194 peaks differ by a factor of 4.7 between a value of 389.20 (sferry) and 1809.58 (olbers). In general, wild types contain higher concentrations and more volatile metabolites than cultivars. Within the *F. vesca* group and also between the group of wild types and cultivars several qualitative differences occur. For example, the key compound ester methyl butanoate is present in all cultivars in high amounts up to a relative concentration of 192.79, however, it amounts to 0.0 in the *F. vesca* accessions 'Island' and 'Boehmen'. The key compound methyl anthranilate is evident in all *F. vesca* accessions but has been observed only in the cultivars 'Mieze Schindler' und 'Mara de Bois' in moderate amounts. The concentrations in *F. vesca* accessions vary in a wide range of a factor 100 between values of 9.19 (accession no. 8, 'island') and 980 (accession no. 16, 'olbers').

For visualization, a principal component analysis is displayed in Fig. 2. Both groups, *F. vesca* accessions and *F.* × *ananassa* cultivars, are well separated in two stretched clusters in a 'V'-shape (score plot, Fig. 2 left). The 'V'-shape is caused by different concentration levels of the total VOCs with low amounts at the bottom and high levels at the edges (quantitative differences). The cultivars are grouped in one cluster and the *F. vesca* accessions create a line on the left side. Different compositions of the VOC patterns (qualitative differences) result in an angle between the *F. vesca* and the cultivar cluster in the visualization.

The parameter plot (Fig. 2 right) including 194 peaks values reflects the clustering of the score plot on the metabolite level. From the location of typical volatiles the composition of corresponding genotypes can be identified. Therefore, accessions close to the bottom are rich in C6 compounds like hexanol, hexenal and (*Z*)-3-hexenol (LOX compounds). *F. vesca* accessions in quadrant 2 of the score plot are characterized by high concentrations of methyl anthranilate, DMHF (furaneol) and especially terpenoids. In contrast, the cultivars in quadrant 1 of the score plot are rich in straight and branched esters, lactones and the terpenoid linalool. Interestingly, *F. vesca* accessions do not contain 2-branched esters which are products of a biochemical pathway starting from amino acids. These esters are powerful aroma compounds with typical fresh-fruity note for instance also found in apples. A typical volatile in ripe *F. vesca* fruit is methyl anthranilate. It is a product of the shikimate pathway synthesized via chorismate [3, 15]. Methyl anthranilate varies within the sixteen *F. vesca* accessions by three orders of magnitude. This compound is characterized by a typical sweetish-flowery smell reminding on acacia. In addition to other volatiles, methyl anthranilate causes the unique flavor of *F. vesca* and the sensory quality of only few *F.* × *ananassa* cultivars like 'Mieze Schindler' (MS) and 'Mara de Bois' (MDB).

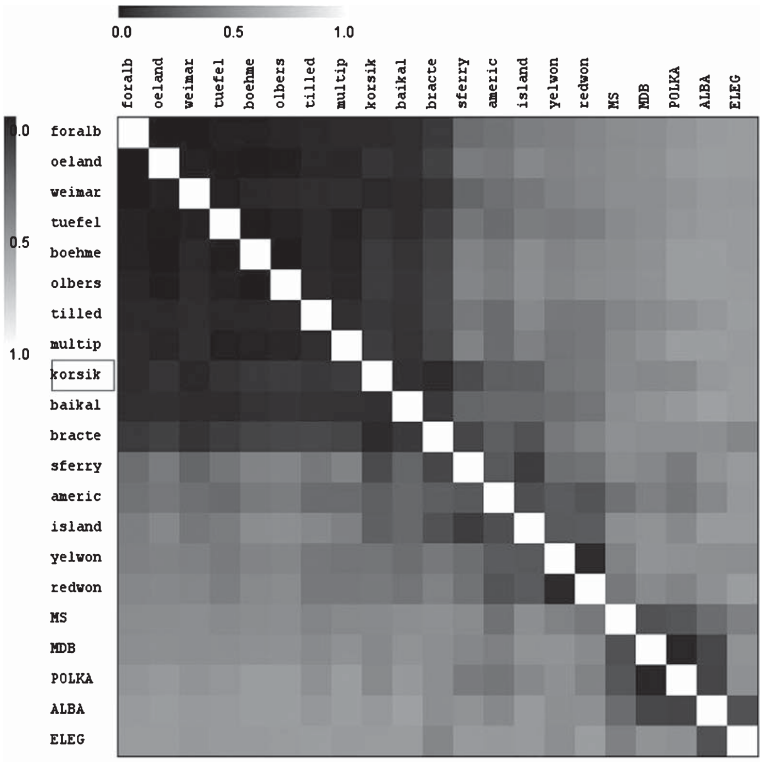

Fig. 3. Distance matrix plot calculated for 194 volatile organic compounds. Color code: black = distance 0 and white = distance 1.

To illustrate the relationship of different genotypes regarding the volatile patterns the matrix of a distance analysis is shown in Fig. 3. The metabolite distance regarding all 194 peaks is presented in a graduation code of grey: the darker the grey value the lower the distance or rather the more similar the volatile patterns of two genotypes. In the upper left corner 11 *F. vesca* accessions are located with a quite high similarity in the aroma pattern. Also the accession 'island' and 'sferry' carry a low distance. The high similarity between the two accessions 'yelwon' and 'redwon' might be a result of the close genetic relationship. 'Red Wonder' (redwon) and 'Yellow Wonder' (yelwon) are both cultivars differing in a fruit color (anthocyanin biosynthesis) mutation. The five *F.* × *ananassa* cultivars form an own cluster at the bottom of the matrix. The cultivars 'Mara de Bois' (MDB) and 'Polka' (POLKA) are also characterized by very similar aroma patterns, especially, with regard to the short chain esters content.

F. vesca accessions produce higher amounts and a higher diversity of terpenoid-derived compounds in comparison to *F.* × *ananassa* cultivars. Terpinen-4-ol, myrtenal, α-terpineol, myrtenol and linalool (identified by mass spectrometry) carry important bioactivities like attractants, pheromones, kairomones, fungicides etc. (Pherobase. www.pherobase.com 2013). The lack or loss of terpenoids in cultivated strawberries may coincide with a loss of resistance to different diseases and herbivores. This causal relationship is rarely investigated so far. Additionally, the low odor thresholds of this compound group have an impact on sensory characteristics [11, 12]. The amounts of terpenoids are generally higher in wild accessions. This may have a negative influence on the sensory quality due to a turpentine-like, herbaceous, woody odor [1]. In the same way, Ulrich et al. [16] described the astringent characters found by descriptive sensory tests in comparison of wild strawberries from Europe and North America with the standard cultivar *F.* × *ananassa* cv. 'Elsanta'. The terpenoid linalool appears as an exception with its pleasant fresh-flowery, citrus-like odor. Linalool was found in higher amounts in cultivars as also described by Chambers et al. [5]. These authors postulated a mutation in the nerolidol synthase (*FaNES1*) occuring in octoploids (*F.* × *ananassa*) in contrast to the diploids (*F. vesca*). In contrast to the findings of Chambers et al. we could detect linalool concentrations

in *F. vesca* although they are low. This result may support the interpretation that linalool synthesis may arise also from an alternative biosynthesis other than *FaNES1*.

4. Conclusions

High diversity in between 16 accessions of *F. vesca* from the most far east habitat at Lake Baikal, through Europe till North America could be detected by volatile analysis. The group of *F.* × *ananassa* cultivars is clearly separated regarding their aroma patterns.

The results presented give interesting insights in strawberry metabolic diversity and may be useful for further breeding experiments with wild types aiming at excellent flavor notes. The diploid wood strawberry *F. vesca* became a model plant for molecular studies in the *Rosaceae* family. The detected metabolic differences within *F. vesca* can be used for further investigations of biosynthetic pathways or transcriptomics or even marker development.

The isolation method for plant volatiles by imm-SBSE in combination with a laborious berry sampling strategy for ripe and healthy berries was used to prevent temporary influences which occur between different harvest dates within the whole season. Taking into account that changes of metabolite contents between different harvest dates within one season may arise in the same order like genotype differences [9], this kind of sampling is an essential prerequisite for the assessment of genetic diversity of metabolic patterns unbiased of influences of the harvest date. On the other hand, this sampling strategy is smoothing metabolite differences occurring at single harvests. Therefore, this method is not suitable for instance for transcription studies in which the actual metabolic status has to be considered.

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