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Fruit flesh volatile and carotenoid profile analysis within the *Cucumis melo* L. species reveals unexploited variability for future genetic breeding

Running title: Fruit flesh volatile and carotenoid profile variability within the *Cucumis melo* L. species

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ABSTRACT

BACKGROUND: Aroma profile and carotenoids content of melon flesh are two important aspects influencing the quality of this fruit that have been characterized using only selected genotypes. However, the extant variability of the whole species remains unknown.

RESULTS: A complete view of the volatile/carotenoids profiles of melon flesh was obtained analyzing 71 accessions, representing the whole diversity of the species. Gas chromatography coupled to mass spectrometry (GC/MS) and HPLC were used to analyze 200 volatile compounds and 5 carotenoids. Genotypes were classified in two main clusters (high/low aroma), but with a large diversity of differential profiles within each cluster, consistent with the ripening behavior, the flesh color and the proposed evolutionary and breeding history of the different horticultural groups.

CONCLUSION: Our results highlight the huge amount of untapped aroma diversity of melon germplasm, especially of non-commercial types. Also, landraces with high nutritional value regarding carotenoids have been identified. All this knowledge will encourage melon breeding, facilitating the selection of the genetic resources more appropriated to develop cultivars with new aromatic profiles or to minimize the impact of breeding on melon quality. The newly characterized sources provide the basis for further investigations into specific genes/alleles contributing to melon flesh quality.

Keywords: aroma, volatile compounds, melon, diversity, quality breeding, carotenoids

INTRODUCTION

Melon (*Cucumis melo* L.) is one of the most economically important crops worldwide, mostly consumed as a dessert fruit, although some types are used as vegetables. This species has been traditionally divided into two subspecies (*melo* and *agrestis*)[1] and shows great variation in morphological, physiological and organoleptic traits[2]. One of the most accepted intraspecific classification[3] presents 13 groups: *cantalupensis* Naudin and *reticulatus* Ser. often considered the same group (cantaloupes, muskmelons), *inodorus* H.Jac. (winter melons, casaba melons), *ameri* Pangalo (Asian melons), *flexuosus* L. (snake melons), *chate* Hasselq. (cucumber melons), *dudaim* L. (pocket melons) and *tibish* Mohamed within subspecies *melo*; and *acidulus* Naudin, *momordica* Roxb. (snap melons), and the groups *conomon* Thunb., *makuwa* Makino and *chinensis* Pangalo (pickling melons), which sometimes are referred as group *conomon*, within subspecies *agrestis*. Nevertheless, a new classification comprising 19 groups has recently been established[2], and types like *tibish* and the feral American *chito* have been reclassified into subspecies *agrestis* according to molecular studies[4,5].

Although every melon market class has their specific breeding objectives, a common goal nowadays is to achieve high quality standards regarding organoleptic and nutritional properties like sweetness, aroma, and vitamin and antioxidant contents. Aroma, along with sugar and acid content, is the most important factor contributing to melon quality as perceived by consumers, and is dictated by the content of several volatile organic compounds (VOCs) of diverse chemical nature whose levels are regulated during fruit ripening through complex biosynthesis pathways and/or accumulation processes. The differences in VOC profile are dramatic between species, but can also be noticeable within species.

Most of the VOCs reported in *C. melo* are esters (40%), aldehydes (18%) and alcohols (11%), although other types of compounds have also detected in small amounts like terpenes, apocarotenoids, thioesters, lactones, furans, phenolics and sulfides (reviewed by Gonda *et al*.[6]). To date, the literature has revealed more than 300 volatiles in different melon genotypes, with important differences in the volatile profiles of climacteric (the aromatic ones) *versus* non-climacteric (non-aromatic) types[7,6]. In the climacteric melons VOCs are mostly esters, which are compounds with low-odor thresholds usually providing fruity notes, but also sulfur-containing compounds, short-chain alcohols, aldehydes and norisoprenes. Non-climacteric melons are characterized by the lack of esters. In these genotypes aldehydes and alcohols are more abundant. In fact, it has been suggested that the aroma profile is highly genotype-dependent, although it also presents environmental effect[8]. Most studies conducted to date in melon have analyzed some genotypes of commercial interest, mainly representatives of the cantaloupe or winter melon market classes, with only a few references including samples of other horticultural groups (*conomon, makuwa* and *dudaim*)[9-14,8,15-21]. These previous studies have focused on specific chemical pathways or on the changes in the VOC content measured with different methodologies, and under different developmental stages, postharvest conditions, or crop management, including fertilization, irrigation, and grafting[10-12,22-26]. Recent advances in melon genome/transcriptome knowledge have been also used to study the genetic basis of the aroma production by associating melon phenotypes with genotypes and gene expression[27,6]. These studies have been performed using selected melons with contrasting phenotypes among those previously characterized, and segregant populations, but the whole variation in this species has not been reported to date.

Most volatiles derive from different phytonutrient compounds[28,29] such as fatty acids, amino acids, phenolics, terpenoids, and also carotenoids, metabolites that are responsible of the red-orange-yellow flesh colors and whose cleavage give rise to apocarotenoid volatiles[30,31]. This organoleptic trait, flesh color, presents high diversity in the species, and its correlation with β-carotene content has been reported[32]. Although several studies have analyzed the carotenoid content in melon flesh of specific cultivars[33-35] or of landraces from specific regions[36], little information is available about the diversity of carotenoids in large germplasm collections that represent the variability of the entire species.

In this context, the goal of this work is the characterization of the aroma and carotenoid profiles of the melon species, using the largest and more representative melon collection analyzed to date. This melon core collection was previously selected to cover the botanical, geographic, agronomic and phenotypic diversity, and the molecular variability of this crop. It includes not only commercial types, but a large number of landraces and wild melons. The results, consistent with taxonomic and ripening classifications, show new profiles in some European, Asian and African landraces, studied here for the first time and that are in the origin of the most economically important horticultural groups. This information will encourage future genetic studies of specific profiles and the use of the most promising types for melon breeding.

MATERIAL AND METHODS

Plant material

A total of 71 accessions belonging to the melon core collection maintained at COMAV-UPV (Institute for the Conservation and Breeding of Agricultural Biodiversity of the Universitat Politècnica de València, Spain) were selected for the study of the fruit flesh

aroma profile. They represent diverse origin and taxa, both subspecies and all the horticultural groups, and are highly diverse according to the extensive genotyping and phenotyping (seed, vine, flowering and fruit traits) performed in previous studies[4,5]. They also represent diverse breeding status, including cultivated (commercial and landraces), feral and wild forms. A subset of 43 genotypes was also evaluated for their carotenoid content (SI Table 1).

Sample preparation and analysis conditions

Cultivation was performed under common greenhouse conditions in Valencia (Spain), at COMAV´s facilities (February to July 2012). Three plants per accession were grown in a randomized design and fruits were collected when mature, depending on the climacteric or non-climacteric behavior.

For volatile analysis, juice was squeezed, filtered with gauze and treated with saturated calcium chloride solution as described by Obando-Ulloa *et al.*[10]. The aliquots of the resulting mixture were stored at -80ºC until analysis. A total of 206 samples (from the 71 selected accessions) were analyzed, since a few plants did not bear fruits or they were collected still unripen. Immediately before analysis, samples were thawed at 30°C for 5 min, and 1 ml was transferred to a 10 mL screw cap headspace vial. Volatile compounds were captured by headspace solid phase microextraction (HS-SPME), and separated and detected by gas chromatography coupled to mass spectrometry (GC/MS). Volatile extraction was performed with a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber (Supelco). Samples were first tempered at 50ºC during 10 min, and then the fiber exposed to the headspace for 20 min at 50ºC. Desorption was performed for 1 min at 250ºC in splitless mode. Automated sample preparation and injection were performed with a CombiPAL autosampler (CTC Analytics).

Chromatographic separation and detection were performed in a 6890N gas chromatograph coupled to a 5975B mass spectrometer (Agilent Technologies) with a DB-5ms capillary column (60 m, 0.25 mm, 1 μm) (J&W Scientific). Oven programming conditions were 40ºC for 2 min, 5ºC/min ramp until 250ºC, then 250ºC for 5 min, with a 1.2 mL/min Helium constant flow. Electronic impact ionization (70eV) was used at 230ºC ionization temperature. Acquisition was performed in scan mode with *m/z* mass range 35-220 (seven scans/s).

Untargeted analysis was performed with MetAlign software (WUR, http://www.metalign.nl). For quantitation, one specific ion was selected per compound. An admixture reference sample was prepared by mixing thoroughly equal amounts of each sample. An aliquot (1 mL) of this admixture was analyzed after every five, and used as a reference to correct for temporal variation and fiber aging. The normalized results were expressed as the ratio of the abundance of each compound in a particular sample to that present in the reference admixture.

Compound tentative identification was performed by comparison of its mass spectrum with that in the NIST05 Mass Spectral Database. When available, mass spectral identity and coelution with pure standards (Sigma-Aldrich) were used for unequivocal compound identification.

For carotenoid analysis, fruit flesh samples extracted as cylinders from the equator of the fruit were stored at -80ºC and subsequently lyophilized for its use. A total number of 79 samples corresponding to the 43 accessions selected were analysed (2 samples per accession in most cases). Extraction of total carotenoids was conducted following the protocol described in Ibdah *et al.*[30] with some modifications. Carotenoids were extracted from 250 mg of the lyophilized material with 5 mL of extraction solvent (hexane/acetone/ethanol:2/1/1), sonicated for 10 min and immediately centrifuged at

10000 g during 15 min at rt. The supernatant was then saponificated during 30 min with 1 mL of 60% KOH followed by two liquid/liquid extractions with 4 and 1 mL of hexane respectively. Organic phase, containing the carotenoids, was dried at 34 °C with a nitrogen flow. The residue was dissolved in 400 µL of a mixture of inject solvent (MeCN/MeOH/CH_2Cl_2: 45/5/50) and passed through a 0.45-µm Nylon filter for HPLC. Twenty µL aliquots were injected into 2996 Waters HPLC equipped with 996 Waters PDA detector and using a YMC-Pack C30 (Tecknokroma) column (250 x·4.6 mm i.d.; 5 µm). The column was equilibrated in 95 % solvent A (MeOH/H2O: 97/3 containing 3.85g ammonium acetate, 680µL of triethylamine, and 1 g BHT) and 5% of solvent B (t-butyl methyl ether containing 680µL of triethylamine, and 1 g BHT). For carotenoid separation the following gradient at a 1 mL/min flow rate was applied: 5% B during 12 min, 5 to 14% solution B within 20 min, achieving 25% B in 30 min, 50% B in 50 min, 75% B in 70 min, and finally 90% B in 82 min. Then the column was washed with 90% B for 2 min and equilibrated with 5% solution B for 8 min before next injection. Detection of carotenoids was performed between λ 260-600 nm, and data were analyzed using the Millenium software. Quantification of β-carotene, lutein, β-cryptoxanthin, lycopene and zeaxanthin was performed with commercially available standard compounds (Extrasynthese).

Data processing and statistical analysis

Hierarchical Cluster Analysis (HCA) was used to analyze the volatile profiles of each genotype (based on average values of 3 replicates). The average ratio of levels of each volatile was log 2 transformed for normalization. Acuity 4.0 software (Axon Instruments) was used for HCA and the Heat map, with distance metrics based on Pearson correlation.

A correlation VOC network was also constructed using Pearson correlation coefficient with the ExpressionCorrelation plug-in for Cytoscape software v2.7.0[37].

Principal Component Analysis (PCA) was carried out with the carotenoid dataset using Statgraphics Centurion XVI and visualized employing CurlyWhirly software. Pairwise Pearson correlation between carotenoids and apocarotenoid volatiles was calculated, as well as a simple factor ANOVA to determine the effect of each carotenoid detected on fruit flesh color. Significant differences between groups were calculated by LSD method 95%.

RESULTS AND DISCUSSION

Identification of volatiles in melon flesh

In order to obtain a volatile profile of the melon fruit flesh as complete as possible, a large core collection of diverse samples was analyzed (71 accessions). The untargeted analysis of the chromatograms allowed the detection of a total of 200 volatile compounds. Comparison of mass spectra and retention time with those of pure standards allowed the unequivocal identification of 69 of them. Additionally, a tentative identification based on mass spectra similarity was provided for another 62 compounds. For the remaining 69 volatiles, a name or at least a plausible chemical structure could not be provided, and remained as unknown. The whole set of VOCs detected in the melon core collection are presented and classified according to their chemical nature into 9 groups in SI Table 2 (A, B).

Correlation between volatile compounds

The HCA performed (Fig. 1) revealed that volatile levels correlated with other volatiles basically according to their chemical structure or biosynthetic pathway, as previously

described in other species[37,38] and also in melon[27]. Seven VOC clusters were formed (Fig. 1, SI Fig. 1), and an enrichment of certain type of compounds in each cluster was observed. The esters were the most diversified and frequent class and clustered into several groups according either to the alcohol (methyl, ethyl, propyl, butyl or other esters) or the acyl CoA precursor (acetate or other). Cluster 1 can be divided into four subclusters (SI Fig. 1). The most different one was mainly composed by sulfur compounds, while the other three subclusters were characterized by their enrichment in esters, mainly ethyl esters. This cluster also included most of the branched-chain amino acid (BCAA) related compounds. Clusters 2, 3 and 4 were characterized by the abundance of acetate esters, while cluster 5 presented a mixture of compounds with esters, but also with phenolic and sulfur- and lipid-derived compounds. Cluster 6 was rich in ethyl esters and also included their alcohol precursor, ethanol, several apocarotenoids, and the only sesquiterpene detected (α-farnesene). Cluster 7 contained most of the lipid-derived compounds and also several apocarotenoids.

The network correlation analysis is shown in Fig. 2. Only strong correlations ($r > 0.85$) are shown in a network defined by 107 nodes and 494 edges (all positive correlations). VOC clusters according to compound family could be observed conforming interconnected metabolite groups (acetates, methyl esters, ethyl esters, propyl/butyl esters, BCAA-related and lipid-derived compounds). The most interconnected groups were the acetate and ethyl ester clusters, displaying high correlations. This result was expected since Freilich *et al.*[27] also found high correlation between these two metabolite groups studying a recombinant inbred line (RIL) population derived from two climacteric genotypes. In our study, performed with the whole species variability, also non-climacteric types, additional correlations (although lower) were found between the acetate group and methyl, propyl and butyl esters, while many of these compounds were

not present in Freilich´s RILs[27]. Acetates derived from long-chain alcohols clustered together but were not interconnected with other acetates. In addition, this analysis allowed us to classify some unknown compounds to as belonging to specific volatile groups.

The volatile profiles as biomarkers characteristic of specific melon genetic resources

According to the volatile profiles in the fruit flesh, the different genotypes were classified by HCA in two major clusters: one grouping most of the aromatic accessions (cluster I) and the other the non-aromatic or low aroma accessions (cluster II) (Fig. 1). Only a few VOCs were present in most accessions in similar amounts (SI Fig. 1), including methyl esters like methyl benzoate, lipid-derived aldehydes like (Z)-2 nonenal or ketones like 3-octanone, phenolics like benzaldehyde, and some sulfur compounds. We next analyze in more detail the different clusters of genotypes produced according to their volatile profiles. Images of some fruits of the most representative assayed accessions are compiled in SI Fig. 2.

<u>Aromatic melon genotypes (cluster I)</u>

This cluster, rich in esters (mostly ethyl esters, known to be associated to the typical melon aroma), represented different volatile profiles. Most of the accessions in cluster I were sweet aromatic melon cultivars of the subspecies *melo* (*cantalupensis-reticulatus*, *ameri*), but there were also a few exotic landraces of the *conomon* (subspecies *agrestis*) and *dudaim* (taxonomically classified within subspecies *melo*, but intermediate between both subspecies according to molecular studies) groups.

Cantalupensis-reticulatus and *ameri* melons (I-Ia and I-II):

249 *Cantalupensis-reticulatus* group is along with *inodorus* the horticultural group that
250 includes most of the commercially important cultivars. There exist different market
251 classes, being Charentais, American Western, Prescott, and Ogen among the most
252 important[2]. Our results indicated that there is more variability than expected according
253 to previous studies within the *cantalupensis-reticulatus* group. In fact, four groups of
254 these melons could be distinguished according to their volatile profile.
255 Aroma of orange-fleshed French Charentais melons, such as Vedrantais and Nantais
256 Oblong, was similar to that of Israelian and Japanese cantaloupes with green/light
257 orange flesh, such as Dvash Ha Ogen and Pearl (I-Ia$_1$ in Fig. 1). This group had the
258 highest levels of ethyl esters among the assayed melons, with also high levels of propyl
259 and butyl esters, and also acetates. The Japanese cultivar (Pearl) had less BCAA-related
260 compounds and specific methyl esters and acetates, and also a different pattern of
261 apocarotenoids. This group was quite similar to the French and American Western
262 melons, Dulce and Top Mark and to the *ameri* Ananas (I-Ia$_2$), except for the fact that
263 these latter had less sulfur volatiles and more phenolic compounds, such as benzyl
264 alcohol, 2-phenylethanol or benzaldehyde. Apart from these commercial types, this
265 second group included the Portuguese landrace Casca de Carvalho, highly appreciated
266 by its intense aroma and taste. Aroma profile agrees with previous molecular data that
267 classifies these two groups of climacteric melons in different populations according to a
268 SNP-based STRUCTURE analysis[5].
269 Most of the accessions clustered in I-Ia$_1$ and I-Ia$_2$ are reference cultivars used to
270 characterize melon aroma in previous studies[15,16,27,24]. Our work includes many more
271 cultivars and accessions, analyzed here for the first time, which show aroma profiles
272 different from these standards. A third group (I-Ia$_3$) included *cantalupensis* varieties
273 from Bulgaria and Japan, (Ogen and Earl´s favourite), and the *ameri* Ananas Yokneam.

In this aromatic group a set of traditional Spanish landraces, not previously analyzed, was included (Bolas, Común and Eriçó). Despite most of the Spanish landraces belong to the non-climacteric, non-aromatic *inodorus* group, these landraces are slightly climacteric and in previous phenotyping assays were sensorially classified as with medium aroma (SI Table 1). The main differences of I-Ia$_3$ with the previous groups were the presence of more sulfur compounds and specific acetates, and significantly lower amounts of some ethyl esters (ethyl heptanoate, ethyl octanoate, ethyl dodecanoate). This lower content of these ethyl esters is a common feature with accessions included in the fourth group (I-IIa) mostly composed of French and Italian heirloom *cantalupensis-reticulatus* landraces, Kroumir, Petit Gris de Rennes, Zatta, and the American Western, Golden Champlain and Hale Best Jumbo, which are similar to several Eastern Europe and Central Asian *ameri* varieties (I-IIb). Cantaloupes of I-IIa were enriched in some nitriles, some particular acetates, like methyl acetate, and apocarotenoids, like geranylacetone and β-ionone whereas the *ameri* accessions of I-IIb were richer in sulfur compounds, and in propyl and butyl esters. The *ameri* group is considered one of the oldest and most variable from which most of the current climacteric melons could have derived. The aroma of these *ameri* melons has not been studied in detail previously. The large collection used here allowed the identification of a sulfurous profile in the *ameri* group. Previous studies indicated a high amount of sulfur compounds in the *momordica* (subspecies *agrestis*) group[15], which probably indicates an early derivation of *ameri* melons from the *momordica* types.

Conomon and *dudaim* melons (I-Ib):

As mentioned before, the cluster I, grouping the most aromatic melons, included all *dudaim* and a few accessions of the *conomon* group (those belonging to the *makuwa* type according to Pitrat[2]). These exotic accessions are low to medium sugar and have an

299 intermediate climacteric behavior[5]. According to our aroma profile, *conomon* and
300 *dudaim* varieties were poorer than sweet climacteric melons in propyl and butyl esters,
301 and also in some specific acetates like 2-methylpropyl acetate (floral notes), 2-
302 methylbutyl acetate (vegetable and banana notes), butyl acetate (grape-like notes). The
303 classification of these *makuwa* types of the *conomon* group within this aromatic cluster
304 was not unexpected, as *makuwa* melons have been reported to have a specific aroma
305 and climacteric behavior. Several recent studies reported esters, basically acetates and
306 ethyl esters, in this sweet oriental *makuwa* melons, detecting ethyl acetate and hexyl
307 acetate as the principal volatile constituents, which is in agreement with our results[19-21].
308 Molecular results also agree with this similarity, as previous studies grouped some
309 *makuwa* types with cantaloupes[5]. The remaining *conomon* accessions analyzed in this
310 study grouped with *inodorus* in cluster II, but the aromatic *makuwa* shared with them
311 the high content in α-farnesene (a principal constituent in apple skin), the only
312 unequivocally identified sesquiterpene, suggesting that it could be a discriminant
313 volatile for this group. The scarcity in sesquiterpenes in melon flesh is consistent with
314 previous studies that revealed their major importance in rind[39,40].
315 The *dudaim* group is considered as the one with the strongest and more singular aroma
316 within melons, and it is mainly used for ornamental purposes [9,41]. Their singular external
317 and internal aroma is also perceived by olfaction (see SI Table 1). The flesh aroma
318 profile in Queen Anne´s Pocket Melon (a classical variety of the *dudaim* group), was
319 studied in detail by Aubert and Pitrat[9] finding several lactones, including γ-
320 dodecalactone (a compound abundant in peach and strawberry), that was considered an
321 important possible contributor to the particular aroma of this genotype, as it has a low
322 odor threshold[42]. Additionally, eugenol (a compound with clove-like aroma) and 3-
323 methylbutyl acetate were the volatiles most abundant in this genotype. Our results

indicate that *dudaim* flesh aroma is similar to that of the *makuwa* accessions, but with a remarkable higher content of some monoterpenoids and an specific high content of γ-dodecalactone, and also an unusual high level of eugenol, thus confirming these previous results. However, according to our results obtained by assessing more germplasm, these compounds were not unique in *dudaim,* as the *momordica* MR-1 and the Bulgarian landrace Ogen presented also high amounts of them.

Results of cluster I reveal a variability of "aromatic" melons higher than that previously reported and group commercial melons, landraces and exotic types according to their aromatic profile. This information will facilitate the use of this unexploited germplasm in breeding programs, it will allow, for example, the selection of the sources more appropriated for breeding commercial melons without altering their specific volatile profile or for developing new varieties with differential aroma.

Non-aromatic or low aroma melon genotypes (cluster II)

Most melons considered non-aromatic or with low aroma were grouped in Cluster II, characterized by a low level of total volatiles, and specifically by a low ester content. This cluster included mostly non-climacteric melons, both sweet (*inodorus* varieties) and non-sweet melons (cultivated, *conomon*, *acidulus* and *tibish,* and wild type *agrestis*), but also a few climacteric, although low or non-sugar, genotypes (some *ameri*, *flexuosus*, *chate* and *momordica*) that shared its volatile profile with non-climacteric melons. Cluster II was divided into two subclusters (II-I and II-II) that mostly differed in the amounts of acetate esters and lipid-derived alcohols and aldehydes, compounds responsible of "green leaf" flesh aroma[6].

Non-climacteric sweet *inodorus* and non-sweet *conomon* melons (II-Ia and II-Ib):

The first subcluster (II-I) had in general less lipid-derived compounds and higher acetate esters content than subcluster II-II and included mainly *inodorus* landraces (II-Ia), and

most of the remaining oriental *conomon* (II-Ib), low aroma varieties quite different of the *makuwa* types classified with the aromatic melons. The *inodorus* landraces were mostly Spanish winter or casaba types not usually found in the commercial chain (some traditional Tendral, Blanco and Amarillo landraces), and the international variety Tam Dew. Another group of commercial varieties, representing Spanish melons, including the main commercial "Piel de sapo" market class, grouped in subcluster II-II. Both traditional and commercial *inodorus* melons had similarities in the lipid-derived profile, with moderate levels of linoleic acid derivatives such as pentanal or hexanal, which were less frequent in aromatic melons[8]. Also *inodorus* landraces shared with commercial *inodorus* their low levels of ethyl, butyl and other esters, some acetates like decyl acetate, and alcohols like 2–methylbutanol, abundant in climacteric varieties. However, despite their common origin and the molecular similarities[4], traditional *inodorus* were richer than commercial *inodorus* in some sulfur compounds, in many acetates and methyl esters that were abundant in climacteric, *cantalupensis* and *ameri* melons, and were also present in *momordica.* Our results are in agreement with previous studies[10,18] with the fact that commercial *inodorus* lack some of these volatiles, but also support a new idea that an unexploited variability exists and some *inodorus* landraces present more climacteric-like VOC profiles. Some of these landraces might be used in future breeding programs to develop *inodorus* commercial types with a different aromatic profile.

Climacteric low and no sugar *ameri*, *momordica*, *flexuosus* and *chate* melons (II-IIa):

The second subcluster II-II includes a specific group of Asian varieties of the *ameri*, *momordica*, *flexuosus* and *chate* groups (II-IIa). Despite their climacteric nature, they had medium ester aroma profiles. Our results indicated that these genotypes combine the high lipid-derived profile of wild and exotic *agrestis* types (II-IIc) with a moderate

374 to high content in ethyl esters, like ethyl 2-methylbutanoate or ethyl butanoate, and in
375 some acetates, like hexyl acetate, 2-methylbutyl acetate, butyl acetate and propyl
376 acetate, which are more abundant in cantaloupe melons, and associated to pinneaple,
377 banana, pear, cherry and strawberry sensorial attributes[6]. However, their ester volatile
378 profile was much simpler than that of cantaloupes.
379 This group of accessions was also characterized by a higher abundance of sulfur
380 compounds. These volatiles are important contributors to the distinctive aroma of melon,
381 especially in *momordica*[15]. They are also common in Asian *ameri* landraces (reported
382 here). Our results confirmed that even when this specific pattern is not frequent in the
383 *agrestis* types, it can be found in some wild Indian or African *agrestis*. This type of
384 volatile profile seems to be intermediate between those of climacteric and non-
385 climacteric melons, which is consistent with the high molecular diversity and the
386 intermediate position between both subspecies *melo* and *agrestis* accessions reported for
387 the *ameri*, *momordica* and *flexuosus* groups[4,5].
388 The *momordica* group had also high levels of other compounds, less common in other
389 *agrestis* varieties, but present in some specific groups of aromatic cultivars of the
390 subespecies *melo*. For example, some acetates like ethylphenyl acetate (abundant in
391 cantaloupes and *ameri* groups), and monoterpenes such as the eucalyptol (associated
392 with fresh or minty sensorial attributes), that were detected in *ameri* and cantaloupe
393 varieties and in some specific white type Spanish landraces. *Momordica* varieties were
394 also rich in apocarotenoids, like geranylacetone and β-ionone, which also appeared in
395 most of the orange-fleshed aromatic *cantalupensis*, and in β-damascenone, which was
396 present in most *agrestis*. This is a potent odorant which was firstly isolated in Bulgarian
397 rose oil, but that also has been reported to be important in raspberries and strawberries.

Therefore, the *momordica* group seemed to have one of the most complex aroma profiles among the *agrestis* group.

Non-climacteric sweet *inodorus* cultivars (II-IIb):

The non-climacteric accessions grouped in this second subcluster (II-IIb) represented the most important market classes of the *inodorus* group: the Spanish cultivars Piel de sapo, Amarillo Oro, Rochet, and the international cultivar HoneyDew. Their *inodorus* volatile profile had some important differences with that of climacteric and non-climacteric genotypes grouped in II-IIa and II-IIc. Commercial *inodorus* still displayed high amounts of some lipid-derived compounds with leaf notes like hexanal, or pentanal. However, they showed significantly lower amounts of some VOCs (1-octen-3-ol, (E)-2-hexenal, (E,E)-2,4-heptadienal, (E,E)-2,4-nonadienal, (E,E)-2,4-decadienal, (E)-2-nonenal) associated to sensory attributes such as almond, green, fatty, leafy or tallowy. Some of these compounds had been previously related with the *inodorus* aroma. For example, (E)-2-nonenal is associated with a strong cucumber-like odor, and is consistently detected in *inodorus* melons as well as being associated to the Honeydew melon fruity odor in sensorial analysis[18]. However, we detected this volatile in small quantities in *inodorus* melons.

Additionally, these *inodorus* varieties had a significantly higher content of a few specific esters, acetates and diacetates, than exotic *acidulus* and *tibish* landraces and wild *agrestis* (II-IIc). Some of these VOCs are related to sweet sensory attributes like floral odor (2-methylpropyl acetate, propyl acetate, phenyethyl acetate), banana-like odor (2-methylbutyl acetate, 3-methylbutyl acetate) or grape-like odor (butyl acetate). The amounts detected, however, were lower than those found in climacteric varieties. In addition, these cultivars had significantly moderate levels of the apocarotenoid β-damascenone, quite abundant in *momordica* types.

Although this commercial *inodorus* are considered to be non-aromatic, their flesh has a soft sweet pleasant melon aroma and not the green aroma of wild types that would be undesirable for consumers. The observed reduction in lipid-derived volatiles and the presence of some esters may account for these differences.

Non-climacteric non-sweet *acidulus, tibish* and wild *agrestis* accessions (II-IIc):

The VOC profile with the highest levels of lipid-derived compounds and the lowest levels of esters and acetates, grouped only accessions of the subespecies *agrestis* collected mainly in Africa and India: *acidulus* and *tibish* landraces, wild small-fruit types and one *conomon* from Japan. The cultivated landraces are generally used in Africa and Asia, and consumed raw like a cucumber in salads or pickled. A profile of "green leaf" volatiles was consistent with the cucumber-like characteristics of these genotypes (non-climacteric, non-aromatic, non-sweet and with white or light green flesh). A set of C9 lipid-derived volatiles which confer a typical cucumber-like aroma such as (E)-2-nonenal, (E)-6-nonenal and (E,E)-2,4-nonadienal, were detected in abundance. Some lipid-derived compounds detected have antibacterial and antifungal properties and could have a role in inhibiting pathogen invasion of plant tissues. Accordingly, some wild *agrestis* and *acidulus* have been reported as sources of resistance to pathogenic fungi[3], and we observed unpleasant odor in some of them (the wild *agrestis* Callosus, Tendelti, and Wild Chibbar, SI Table 1).

Identification and quantification of carotenoids

The carotenoid profile of the 43 accessions (SI Table 1) is shown in SI Table 3. According to previous studies[35,29], the main carotenoid detected in the melon flesh was ß-carotene, although lutein and ß-cryptoxanthin were also abundant. Lutein content was significantly higher in green and yellow-fleshed melons, while green/orange and

orange-fleshed melons showed higher levels of ß-cryptoxanthin and, especially ß-carotene, than the remaining genotypes (Table 1). In fact, contents of ß-carotene and ß-cryptoxanthin were moderately correlated (r = 0.73, Table 2). Our results are consistent with previous analysis in which lutein was either not detected or only found at low concentrations in white-fleshed melons like Piel de Sapo or in the orange-fleshed Vedrantais and Dulce[35]. The fruit with the highest levels of lutein in our assay were that of the landrace Casca de Carvalho (yellow flesh, 49.9 nmol g^{-1}FW), analyzed here for the first time.

In general, cantaloupes showed higher levels of β-carotene as previously described[35]. Remarkably high amounts of this carotenoid were detected in climacteric landraces not usually found in markets like the Italian landrace Zatta (orange flesh, 333.8 nmol g^{-1}FW). Regarding ß-cryptoxanthin, the richest accessions were the previously mentioned Zatta (25.5 nmol g^{-1}FW) and Songwhan Charmi (green/orange, 18.8 nmol g^{-1}FW). These results are in agreement with the high level reported previously in Songwhan Charmi[35].

A Principal Component Analysis (Fig. 3), with components PC1(X) and PC2 (Y) explaining 56.9% total variation, showed the variability detected in the collection based on the carotenoid content. ß-carotene and ß-cryptoxanthin were the carotenoids that most accounted for the variability across PC1, while lutein and zeaxanthin did across PC2. Orange-fleshed melons, including light orange ones, were placed along PC1 according to the level of ß-carotene and ß-cryptoxanthin. In the other edge, cream, yellow, white and green-fleshed melons appeared mixed together, only being separated along PC2.

The correlation between carotenoid and apocarotenoid content in melon flesh was calculated and is shown in Table 2. Apocarotenoid volatiles, like 6-methyl-5-hepten-2-

one and geranylacetone presented significant correlation with β-cryptoxanthin (0.43 and 0.39, respectively) and β-carotene (0.31 and 0.30, respectively). In addition, a compound which was putatively identified as an apocarotenoid based on its mass spectrum (Unknown 40.69), displayed the highest correlation with these two carotenoids (0.66 and 0.64, respectively). This result indicates that high content in these two carotenoids associated to orange color is related to high level of apocarotenoids in the volatile profile, which is consistent with previous studies[30]. In fact, groups of accessions based on their apocarotenoid volatile content (SI Table 3) were mostly consistent with those based on their carotenoid pattern. The 43 accessions assayed for carotenoids were grouped in low apocarotenoid types (LA), apocarotenoid-rich non-aromatic types (ANA, basically II-Ib, II-IIc and *momordica* types Fig. 1) and apocarotenoid-rich aromatic cantaloupes (CA, basically I-Ia, I-IIa Fig. 1).

CONCLUSIONS

This work presents the most complete characterization performed to date of the aroma and carotenoids profiles within the whole *C. melo* species. The analysis of this melon core collection, representing the species diversity, has allowed the identification of candidate volatiles that are likely to be responsible for the differences in aroma among different groups of accessions, not only displaying different ripening behaviors, but also belonging to various horticultural groups. As expected, and previously reported, climacteric and non-climacteric accessions presented important differences in the content of volatiles, with higher production of esters in climacteric aromatic types and more lipid-derived aldehydes and alcohols in the non-climacteric and non-aromatic types. Additionally, the inclusion in our study of new germplasm not previously analyzed, both climacteric and non-climacteric landraces and wild accessions, has

allowed the detection of a large amount of variation, underexploited to date. Many new profiles consistent with the geographical origin and history of the different melon groups are shown. This huge variation, mainly found in non-commercial types, justifies the need of this kind of studies as a first step to preserve and optimize the use of these landraces and wild types to widen the VOC spectrum in commercial types through future breeding programs. The detection of *inodorus* landraces with medium/faint aroma, for instance, could be of interest to improve the non-aromatic current commercial *inodorus*. The diversity detected within *cantalupensis-reticulatus* melons is also remarkable, and useful to diversify aroma profiles of cantaloupe cultivars. In addition, the study of strongly aromatic types such as *dudaim*, reinforces the interest that this ornamental type could have in breeding. Other interesting landraces found include those accumulating high levels of carotenoids, and high levels of carotenoid-derived volatile (apocarotenoids) contents.

Therefore, our results uncover interesting germplasm resources that could be used to introduce different volatile and carotenoid profiles into commercial melons. Additionally, the clustering presented might facilitate the selection of breeding sources with VOC profiles similar to those of the commercial melons to minimize the impact of the breeding process on their fruit quality.

Abbreviations

ANOVA: Analysis of Variance

BCAA: Branched-Chain Amino Acid

COMAV-UPV: Institute for the Conservation and Breeding of Agricultural Biodiversity of the Universitat Politècnica de València

FW: Fresh Weight

GC/MS: Gas Chromatography Coupled to Mass Spectrometry

HCA: Hierarchical Cluster Analysis

HS-SPME: Headspace Solid Phase Microextraction

LSD: Least Significant Difference

PCA: Principal Component Analysis

PDMS/DVB: polydimethylsiloxane/divinylbenzene

RIL: recombinant inbred line

VOCs: Volatile Organic Compounds

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SUPPORTING INFORMATION

SI Table 1. Information of the accessions included in the study.

SI Table 2. Volatile compounds detected in the fruits analyzed. A. Fruits analyzed for flesh aroma profile and VOCs detected. B. Annotation in chemical families of VOCs detected.

SI Table 3. Fruits and accessions analyzed for carotenoid content and the individual/mean results.

SI Fig. 1. Hierarchical cluster analysis and heatmap using flesh VOC data. Detail of the clusters showing the VOCs (cluster1, cluster2-5, cluster6, cluster7). VOC color associated to metabolite family: purple (phenolics), pink (sulfur compounds), maroon (BCAA related compounds), red (lipid derivatives), light blue (acetate esters), dark blue (other esters), light green (monoterpenoids), dark green (sesquiterpenes), orange (apocarotenoids), black (others), and grey (unknown).

SI Fig. 2. Images of some representative fruits of the genotypes included in the assay. Can-VedFran, In-HoneyDewUSA, La-ErizoSp, Am-KizilUzbe, In-TeNinvSp, Con-SCKo, Mom-PI124Ind, In-PsPiñSp, La-CascaPor, La-KroFran, Can-HBJUSA, Flex-AryaInd. La-ZatIta Dud-QAPMGeorg, Ag-TendSud, Chi-VellInd.

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Figure captions

Fig. 1. Hierarchical cluster analysis and heatmap using flesh VOC data (Acuity 4.0 software). Volatile clusters (1-7) are indicated. The heatmap was constructed using the distance metrics based on the Pearson correlation. The red to green range of color indicates the level of each volatile in each genotype, according to the scale below (log 2 transformation of the ratio levels of each volatile in a sample/average of all the genotypes analyzed): light red for the highest values; light green, lowest; black, intermediate.

Fig. 2. Correlation network analysis of the flesh VOC data set (Cytoscape software v2.7.0). The nodes representing volatiles are colored according to the volatile family as indicated in the image. Correlations over 0.85 are indicated. Line thickness indicates correlation strength: the wider the line, the stronger the correlation.

Fig. 3. Principal Component Analysis of the carotenoid dataset (visualized using CurlyWhirly software). PC1(X) and PC2(Y) explained 56.9% total variation. Points of different color indicate the color of the fruits of the genotypes assayed (individual values).

Tables

Table 1. Mean and standard deviation of the carotenoid content for the groups of accessions according to flesh color.

Table 2. Pairwise Pearson correlation calculated for carotenoids and volatile apocarotenoids detected in 43 accessions.