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Climacteric or non-climacteric behavior in melon fruit

1. Aroma volatiles

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Abstract

A near-isogenic line (NIL) SC3-5 and other nine NILs of melon contained introgressions of an exotic non-climacteric accession of *Cucumis melo* ´Shongwan Charmi´ [SC (PI 161375), Conomon Group)] into the non-climacteric Spanish Inodorus type of melon cultivar Piel de Sapo (PS). The NILs exhibited different climacteric behavior and aroma. Fruit from SC3-5 and seven NILs showed a climacteric pattern, while fruit from one NIL, both parentals and the cultivar Nicolás, were non-climacteric. The NILs were compared with the reference aromatic cultivars Fado and Védrantais, which show climacteric behavior with high levels of respiration and ethylene production. The twenty-eight aromatic compounds common to the cultivars and NILs studied defined the aroma profile, which was composed of fifteen esters, six aldehydes, two alcohols, three derived sulfur compounds (methyldisulfanylmethane; methanethiolate; methyl 2-sulfanylacetate) and other three compounds (1,7,7-trimethylnorbornan-2-one; acetone; 2-ethylfuran). On the basis of the total ion count peak area, three compounds (isobutyl acetate; benzyl acetate;

pentanal) allowed the climacteric to be distinguished from the non-climacteric NILs according to univariate analysis. Multivariate analysis of the aroma data on the basis of total ion count peak area separated the aromatic attributes of the climacteric ´Védrantais´ and ´Fado´ melons from the NILs that were closer to their inbred parentals when analyzed by partial least squares regression plus discriminant analysis. In the climacteric reference cultivars or NILs, esters were the predominant volatiles while aldehydes predominated in non-climacteric ones. These results support the hypothesis that at least one QTL in linkage group III boosts a series of maturation signals that are characteristic of climacteric fruit, including a different aroma profile.

Key–words: *Cucumis melo* L.; Nnear-isogenic lines; Eethylene production; Rrespiration rate; Aaroma profile; Qquantitative trait loci; Mmultivariate statistics.

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1. Introduction

The classification of fruit in climacteric and non-climacteric is considered an over-simplification (Obando et al., 2007). Climacteric ripening is characterized by an upsurge in the respiration rate accompanying an autocatalytic ethylene production peak during fruit ripening (Périn et al., 2002; Kays and Paull, 2004). In contrast, non-climacteric fruit ripening presents cyanide insensitive respiration to a lesser extent than climacteric ones, and the upsurge of respiration and ethylene are not observed or are transitory after ethylene application (Lurie and Klein, 1989; Kays and Paull, 2004). Melon fruit (*Cucumis melo* L.) show a high diversity of ripening behavior, including climacteric and non-climacteric genotypes (Flores et al., 2002; Beaulieu, 2005). Commercially, fruit with climacteric

behaviour shows a short shelf-life and display more aroma than non-climacteric fruit, because some aroma compounds are produced only by ethylene-dependent pathways. However, both dependent and independent pathways coexist during melon ripening (Flores et al., 2002).

Around 240 compounds have been identified in climacteric cultivars such as Galia or Cantaloupe melons (about half of them are esters, and most of the remaining components are sulfur-derived compounds, aldehydes and alcohols) (Beaulieu and Grimm, 2001; Fallik et al., 2001; El-Sharkawy et al., 2005; Kourkoutas et al., 2006). In contrast, non-climacteric cultivars shows lower levels of total aroma compounds (42 compounds identified in Hami melons according to Moshonas et al., 1993), and lack of esters (Aubert and Pitrat, 2006; Saftner et al., 2006).

Flavor volatiles are derived from an array of compounds including some phytonutrients such as fatty acids, amino acids, carotenoids, phenolics and terpenoids (Baldwin, 2002; Goff and Klee, 2006). The volatile esters are formed during the esterification of alcohols by the alcohol acetyltransferase (AAT), normally using CoA moiety or CoA-ester as the acyl donor during the ripening of many fruit including melons (Beaulieu, 2006).

Aldehydes arise from the enzymatic degradation of lipids and/or are produced from free fatty acids such as linoleic and linolenic acids via lipoxygenase activity or amino acids (such as acetaldehyde that comes from alanine). Also, specific hydroperoxide lyases acting as isomerases can perform a homolytic rearrangement of fatty acid hydroperoxides into short-lived hemiacetals which upon decomposition produce 3(Z)-nonenal, 3(Z)-hexenal and other short chain aldehydes (Grechkin et al., 2006). Other aldehydes such as the hexanal increases after tissue disruption (Baldwin, 2000 and 2002; Goff and Klee, 2006).

Later, these compounds are reduced to alcohols in a reaction carried out by alcohol dehydrogenase (ADH) (Baldwin, 2002; Zhu et al., 2005; Manríquez et al., 2006). On the other hand, sulfur compounds are likely to be derived from the sulfur-containing amino acids (cysteine/cystine, and methionine), reducing sugars and thiamin (vitamin B_1) (Mussinan and Keelan, 1994; Wyllie et al., 1995).

The knowledge about genetic control of melon fruit quality traits is still scarce (Périn et al., 2002; Monforte et al., 2004). Eshed and Zamir (1994) proposed developing introgression line (IL) populations by marker assisted selection, consisting of a set of lines whereby each one contained a single homozygous chromosome segment from a donor parent in the genetic background of an elite cultivar. Intentionally, these lines have a high percentage (mostly higher than 95%) of the recurrent parent genome, so they are also defined as near-isogenic lines (NILs). The analysis of NIL populations significantly facilitates complex trait or quantitative trait loci (QTL) analysis (Zamir, 2001; Eduardo et al., 2005 and 2007; Fernández-Trujillo et al., 2007).

Eduardo et al. (2005) developed a melon NIL collection from the non-climacteric parentals "Piel de Sapo" (PS, *inodorus* type) and the Korean accession PI -161375. Within this collection, the NIL SC3-5 was aromatic and so was selected to study the link between climacteric behavior and aroma (Fernández-Trujillo et al., 2007; Moreno et al., ~~unpublished~~in press). The objective of this study was to characterize the aroma profile of reference climacteric or non-climacteric genotypes to compare them with climacteric or non-climacteric NILs. The second goal was to compare the aroma profile of the climacteric NILs with the non-climacteric ones to identify the most discriminant volatiles from the profile associated with either behavior.

2. Material and methods

2.1. Plant material

The parental lines used were *C. melo* var. *inodorus* Naud or Spanish cultivar Piel de Sapo (PS) from Semillas Fitó S.A. (Barcelona, Spain), and the exotic *C. melo* ssp. Conomon Group or Korean accession SC (~~PI161375~~PI 161375). The reference climacteric lines used were a commercial ´Galia´ melon (*C. melo* var. *reticulatus* cultivar Fado F1; Semillas Fitó) and the parental *Charentais* type (*C. melo* var. *cantalupensis* Naud, cultivar Védrantais). As a reference of non-climacteric type, a commercial ´Piel de sapo´ type F1 hybrid cultivar Nicolás (Syngenta Seeds S.A., Torre Pacheco) was used. The NIL SC3-5 was derived from a cross between PS and SC which was targeted to contain a unique introgression on linkage group (LG) III (Eduardo et al., 2005 and 2007). Nine additional NILs (5M2 to 5M10) with shorter introgressions of SC on the LG III developed during the process of NIL construction were also used for analysis.

2.2. Experimental design

Melon seeds were planted on March 7, 2005 and transplanted to a seedbed on March 9 in a nursery greenhouse in Torre Pacheco (Murcia, Spain). The NILs with shorter introgressions than SC3-5 were labelled as 5Mx (x=2 to 10). Plants were grown in an open field managed by CIFEA (Torre Pacheco, Murcia, Spain) according to conventional methodology (Fernández-Trujillo et al., 2007; Obando et al., ~~in press~~2008). Each replicate in the field consisted of three plants, except the cultivars SC, Fado and Védrantais, which consisted of individual plants. The replicates were n=20 for PS, n=11 for SC, n=9 for NILs

5M2, 5M9 and 5M10; n=7 for the rest of the NILs and SC3-5, n=8 for ´Fado´ or ´Védrantais´, and n=6 for ´Nicolás´. Fruit were harvested using a combination of different harvest index depending on their climacteric or non-climacteric behavior. Minimum harvest indices were the presence of a well formed and defect-free fruit, firm, well healed and dry epidermis with lignified netting, high density according to previous recall of the harvester, skin color, and stem scar development (Obando et al., 2007). The more common harvest indices were yellowing of the ground spot (in PS), about ½ to ¾ slip or skin netting development (in PS ~~and~~, SC and ~~,~~ 5M7), yellowing close to the slip area (in ´Nicolás´); development of annular ring in the peduncle that precedes fruit dehiscence (in dehiscent NILs, also accompanied by the slip criteria), the start of cracking (visible, but not open) or history of cracking in NIL SC3-5; volatile emission detected by human nose (in ´Védrantais´ and climacteric NILs), light yellow skin color (in ´Fado´), and whole fruit texture and peduncle suberization.

2.3. Respiration rate and ethylene production

Both physiological activities were monitored at 21±1 ºC and 66±6% relative humidity (mean±SD during the season) using the static method in order to measure a possible climacteric peak of respiration and accompanying peak in ethylene production. The experimental procedure and sample analysis by gas chromatography was conducted according to Fernández-Trujillo et al. (2005) and Moreno et al. (in press).

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2.4. Flesh and juice sampling for volatile analysis

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Juice was squeezed with a Simplex Super metal juicer (Italy) using 20 mm long and 15 mm diameter flesh cylinders obtained by an apple cork borer from the middle of one of the longitudinal sections of every fruit (Obando et al., ~~in press~~2008). Samples were taken by filtering juice through a powder funnel and four-layer cheesecloth. After 3 min, 4 mL of a saturated calcium chloride solution were added to 10 mL of juice and the mixture was homogenized, according to Baldwin et al. (2004). An aliquot of the mixture was poured into a 12 mL sterile PP vial (102 × 15 mm; Deltalab S.A., Rubí, Barcelona, Spain). These samples were stored in a freezer at -70 ºC until transportation to the Katholieke Universiteit Leuven (Belgium). Samples were transported (four hours by air~~plane~~) in a 10 mm-thick polystyrene icebox with eight dry-CO_2 tablets of 100 g, and then stored at -80 ºC until analysis.

2.5. ~~Headspace~~ Volatile analyisis: Headspace formation, s~~.~~ ~~S~~olid-phase micro-extraction (SPME), ~~-G~~gas chromatography-~~-~~mass spectrometry (GC-MS) analysis.

The methodology for GC-MS analysis of the samples was based on Berna et al. (2005) and Muriel et al. (2004). Melon juice samples were thawed in a water bath at 35 ºC for 15 min, then an aliquot of 5 mL mixture plus an internal standard (10 µL of phenyl-ethyl alcohol 0.1% v/v, Acros Organics, NJ) was poured into a 20 mL glass vial (Gerstel, Germany). The vials were sealed using crimp-top caps with TFE/ silicone septa seals (Alltech Associates, Inc., Deerfield, IL) and placed in the heat tray of the GC (6890N, Agilent Technologies, Wilmington, DEL) at 35 ºC for 2 h~~ours~~ for the headspace to form.

The 1-cm long SPME fiber, previously preconditioned in the injection port at 250 ºC for 1 h, contained a 50/30 µm divinylbenzene/carboxene on polydimethylsiloxane coating

(57329-U DVB/Carboxen™/PDMS Stable Flex™ Fiber, Supelco, Bellefonte, PA). The needle entered 22 mm into the vial headspace and remained 30 min at 35 ºC absorbing the volatiles. After extraction, the volatiles were desorbed from the SPME fibre into the GC (6890N, Agilent Technologies) injection port set at 280 ºC for 3 min as a bake-out step.

The analyses were conducted with a MPS2 Gerstel Multipurpose sampler coupled to the GC- MS. The injection port was operated at 280 ºC in splitless mode and subjected to a pressure of 80 psi. Volatiles were separated on a 30 m × 0.25 mm id × 0.25 µm thickness capillary column (HP- 5MS, Agilent Technol.) that contained 5% phenyl-methyl silicone as a stationary phase. The carrier gas was helium with a flow rate of 1.5 $mL \cdot min^{-1}$. The initial oven temperature was 35 ºC, followed by a ramp of 2 $ºC \cdot min^{-1}$ up to 75 ºC, and then at 50 $ºC \cdot min^{-1}$ to reach a final temperature of 250 ºC, which was held for 5 min. The inlet liner used was a 2637505 SPME/direct (Supelco), 78.5 mm × 6.5 mm × 0.75 mm. Mass spectra were obtained by electron ionization (EI) at 70 eV, and a spectrum range of 40–450 m/z was used. The detector worked at 230 ºC and in full scan with data acquisition and ion mass captured between 30 and 300 AMU. The total analysis time was 27.5 min.

The chromatograms and mass spectra were evaluated using the Chem-Station software (G1791CA, Version C.00.00, Agilent Technol.). The peaks were identified using a mass spectrometer (5973 Network Mass Selective Detector, Agilent Technol.) coupled to the GC by comparison of experimental spectra with those of the National Institute for Standards and Technology (NIST98, search version 2.0) data bank. The retention times from a series of straight-chain alkanes (C8-C20) supplied by Fluka were used under identical conditions to calculate the Kovats indices for all identified compounds (Kondjoyan and Berdagué, 1996). C6 and C7 were calculated from compounds present in our chromatograms. Results from the volatile analyses were expressed as the percentage of each compound´s integrated

area relative to the total integration of 28 compounds. These compounds were recovered and positively identified within the run time (Muriel et al., 2004; Beaulieu, 2005 and 2006; Beaulieu and Lea, 2006). The Chemical Abstract Service (CAS) numbers of the volatiles reported in the NIST98 database also found in the website http://webbook.nist.gov/chemistry/name-ser.html (Supplementary Table 1), were used for obtaining their corresponding IUPAC names by checking the website http://www.chemindustry.com/apps/chemicals.

To plot the average chromatogram of the forty PS samples, a worksheet was programmed in Matlab ® 6.5.0.180913a Release 13 (The MathWorks Inc., Natick, MA) in order to compute the average of the areas and retention times after getting them from the GC-MS.

2.6. Statistical analysis

The volatile data obtained in the aroma profiling experiments were analyzed with partial least squares discriminant analysis (PLS-DA). This multivariate statistical technique allows clustering and grouping of observations with similar aroma profiles and identification of the original aroma compounds responsible for this discrimination (Baldwin, 2002). The different levels of the response variable – the different cultivars in this case- were replaced by a set of dummy variables describing the categories. Next, the vector of response variables generated was regressed onto the explanatory variables being the aroma volatiles. This results in a set of PLS components, which are a linear combination of the original X explanatory aroma volatile variables. The set of coefficients is defined through an optimization algorithm in the software, maximizing at the same time

the description of the variability of the explanatory variables and the prediction of the response variables (Karp et al., 2005; Gabrielsson and Trygg, 2006). To visualize the multidimensional data structure, score plots and correlation loading plots were constructed. In the score plot the data of the cultivars and NILs were plotted as centroids in a bidimensional space represented by the first two latent variables (PC´s). Each centroid point corresponded to the average value of all the observations within each NIL and was used for an easier interpretation and visualization of the figure.

The correlation loading plot indicates the correlation between the original variables (aroma volatiles) and the PLS-components. The coordinates of an aroma volatile (these are the projections of the aroma volatiles onto both axes) on the first and the second latent variables show how well this volatiles is correlated with the latent variable. The inner and outer ellipses on the figures represent correlation coefficients r=70% and r= 100% (or r-squared values of 50% and 100%). For an aroma volatile located between the two ellipses more than 70% of its variability is explained by the first two latent variables. This means this variable is important in describing the variability, and, hence, the major cultivar effects, within the data set. The analyses were carried out in The Unscrambler software (version 9.0, CAMO A/S, Trondheim, Norway). In addition, the ellipsoid around each centroid was calculated at 95% confidence interval with the ellipse package (Version 0.3-4) in R language (R Foundation for Statistical Computing, Vienna, Austria).

In a univariate context, significant differences were studied by ANOVA using pedigree (SC3-5, NIL codes or PS) as factor. A Dunnett test at P=0.05 was used to determine the differences between NILs and PS. To validate this profile, the PS and reference cultivars volatile levels were compared among them and significant differences after ANOVA were established by a Tukey test at P=0.05. Both analyses were performed with JMP v5.1.2 for

Windows (SAS Institute Inc., NC).

3. Results

3.1. Respiration rate and ethylene production ~~(RR and EP)~~

The melon fruit from the parental lines PS and SC and the commercial cultivar ´Nicolás´ were characterized as having ~~a~~ non-climacteric behavior accompanied by low ~~EP~~ ethylene production and the absence of an ethylene peak at 21 ºC, even though the ~~EP~~ethylene production levels where slightly different among them, particularly at harvest (Fig. 1A and 1C). The cultivar ´Védrantais´ (Fig. 1B and 1D) showed climacteric behavior with ~~RR~~ respiration rate and ~~EP~~ethylene production well defined peaks. ´Fado´, also climacteric (data not shown), showed a typical post-climacteric behavior because was harvested at the light-yellow stage of maturity.

The NILs SC3-5, 5M3-5M6 (5M2 was not monitored) and 5M8-5M10 were climacteric (with respiration rate~~RR~~ and ethylene production~~EP~~ defined peaks), while 5M7 was non-climacteric pattern as PS or SC with low ethylene production~~EP~~. Two main patterns of ~~RR~~ respiration rate and ethylene production~~EP~~ were found among the NILs: the SC3-5 pattern was shared by 5M5, 5M6 and 5M9. The 5M3 pattern (more pronounced) was shared by NILS 5M4, 5M8 and 5M10 (Fig. 1B and 1D).

3.2. Aroma profile

Of the approximately 110 compounds tentatively identified in the experiment, only 28

volatile compounds were present in both the headspace of PS, SC3-5 and the NILs (Fig. 2). The profile had fourteen esters (among which acetate esters were predominant over the non-acetates), six aldehydes, two alcohols, three derived sulfur compounds, acetone, 2-ethylfuran, and 1,7,7-trimethylnorbornan-2-one (camphor) (Supplementary Table 1). All the compounds of the profile were also present in the headspace of the NILs and cultivars analyzed as reference. The presence of these compounds was well reproducible with a coefficient of variation lower than 20% in most cases (data not shown).

3.3. PLS-DA of the aroma composition data

A PLS-DA analysis of all the compounds of the aroma profile gave some interesting information about the differences among climacteric and non-climacteric NILs and the reference cultivars (Supplementary Fig. 1). Subsequently a PLS-DA was applied only on a subset of compounds with a major discriminant power (see section 3.5). The correlation loading plot is depicted in Figure 3 where PC1 and PC2 explain in total 86% of the X-variability. The parental line PS (and the cultivar Nicolás to a lesser extent) was localized at positive PC1 values due to the high concentrations of seven compounds (methanethiolate, acetone, 2-ethylfuran, pentanal, hexanal, 2-phenylacetaldehyde, and decanal) (Table 1, Fig. 3). The parental SC was located in the lower left quadrant of the correlation loading plot and its aroma was negatively correlated to PC1 or PC2 with the relative concentrations of ethyl acetate, ethyl butanoate, benzyl acetate and ethyl 2-methylpropanoate (Table 1, Fig. 3). The most characteristic compounds for the climacteric cultivars ‘Fado´ and ´Védrantais´ were propyl acetate, methyl 2-methylbutanoate and hexyl acetate (Table 1, Fig. 3). The results from the multivariate analysis (Figure 3) and the

univariate analysis (Table ~~3~~1) highly correspond, however, for some volatiles there were discrepancies.

In the multivariate analysis of the NIL data (Fig. 4), PC1 and PC2 explain in total 73% of the X-variability. The PC1 was defined in the negative direction by methyl acetate; propyl acetate; isobutyl acetate, while in the positive direction, it was defined by 6 compounds (methanethiolate, pentanal, hexanal, 2-phenylacetaldehyde, nonanal and decanal. The PC2 was mainly influenced by methyl acetate and propan-2-yl acetate (Fig. 4). The parental line PS and the non-climacteric NIL 5M7 were located in an area towards the right of the graph due to the high relative aroma concentration values of the above reported six compounds (Fig. 4). On the other hand, SC3-5 and NILs 5M4, 5M9 and 5M10, all showing a climacteric behavior, were located either in the central zone or towards the left quadrants of the graph due to propyl acetate (Table 2, Fig. 4). Isobutyl acetate and methyl acetate separated, respectively, 5M4 and 5M6 from other climacteric NILs, while 5M3 and 5M8 were separated from other climacteric NILs due to propan-2-yl acetate (Table 2, Fig. 4). However, the differences other than climacteric versus the non-climacteric NIL 5M7 or PS should be interpreted with great care since most of the cultivars are located around the origin and hence are poorly correlated to the PC1 or PC2 axis (Fig. 4).

The grouping of the different NILs according to climacteric or non-climacteric behavior reflected the results of the PLS analyses using the volatile profile. In fact, two esters (methyl acetate; isobutyl acetate) were the most characteristic of the climacteric NILs (Table 2, Supplementary Fig. 1), and pentanal was responsible for distinguishing the non-climacteric NILs (5M7, PS) and ´Nicolás´ from the rest of climacteric NILs (Table 1 and 2, Supplementary Fig. 1). On the other hand, ethyl butanoate was a characteristic

compound of SC (Table 1, Fig. 3, Supplementary Fig. 1).

3.4. Univariate analysis of individual aroma compounds

The parental line PS showed a total average non-acetate ester content of 2.2% and a total acetate ester content of 13.7%. Ethyl acetate was the predominant acetate ester in PS. The acetate or non-acetate ester content was generally the same or increased in several climacteric NILs compared with PS (Table 2). For example the concentration of isobutyl acetate and methyl 2-methylbutanoate was 7.4-1028% higher in the climacteric NILs (Table 2).

The aldehyde compounds in PS ranged from 0.7-52%. Three of these compounds were present in 40 to 81% lower concentrations in the aroma of the climacteric NILs, while other three increased their concentration (1.2-29.6%) in two NILs of the same group. The two alcohols identified in PS were present at around 0.3-0.9%, while their concentrations were reduced by 16 to 72% in the aroma of the climacteric NILs (Table 2).

The sulfur derived compounds methanethiolate and methyldisulfanylmethane in PS showed concentrations of 0.2-5.2%, while the same compounds were present at 16 to 88% lower concentration in the headspace of the climacteric NILs (Table 2).

The content of the other compounds followed two different trends in the headspace of the NILs. PS showed an acetone content around 2.3%, while it was reduced by 40 to 86% in the climacteric NILs. In addition, the content of 2-ethylfuran in PS was around 3%, but it was around 29-77% lower in the climacteric NILs. The content of camphor of PS was 2.7%, but three climacteric NILs had 2- 68% lower contents, while it was increased in the headspace of the other climacteric NILs and 5M7 (non-climacteric) by around 14-114%

(Table 2).

3.5. Classification of volatile compounds by univariate analysis

The volatile compounds identified in the headspace of NILs were classified according to their power to differentiate the NILs into discriminant and non-discriminant compounds. Three volatile compounds (pentanal; isobutyl acetate; benzyl acetate) were preliminarily classified as good discriminant because the NIL 5M7 did not show statistical differences according to PS, but the rest of the NILs did (Table 2). Four compounds (methyl butyrate; methyl butanoate; ethyl 2-methylpropanoate; methyl 2-sulfanylacetate) were classified as non-discriminant because any NIL showed significant differences compared with PS. The rest of twenty-one volatile compounds of the profile were classified as moderately discriminant when there were statistical differences between some of the climacteric NILs (SC3-5 and 5M3 to 5M10) and the group formed by the non-climacteric melons (PS and 5M7).

3.6. Aroma of the reference cultivars versus PS and NILs

Esters were the most abundant compounds (38-96%) in most of the NILs, SC, ´Fado´ and ´Védrantais´. Among the esters, the acetates are the most important of all (Tables 1 and 2, Fig. 3 and 4), especially hexyl acetate due to its high concentrations allowed to separate ´Fado´ and ´Védrantais´ from the other commercial lines (Table 1, Fig. 3) and from the NILs analyzed (Supplementary Fig. 3 and 4). According to the univariante analysis, the differences between SC and ´Fado´ or ´Védrantais´ were based on the relative concentration of two esters (methyl 2-methylbutanoate and hexyl acetate), three aldehydes

(pentanal, 2-phenylacetaldehyde and nonanal), 2-ethylhexan-1-ol, methanethiolate and acetone without differences between ´Fado´ and Védrantais (Table 1). The relative concentration of ethyl butanoate allowed to separate SC and Védrantais from ´Fado´. Also, methyl butanoate allowed to separate ´Védrantais´ from SC (Table 1, Supplementary Fig. 1).

Regarding the non-climacteric NIL 5M7, PS and ´Nicolás´, the aldehydes were the most abundant compounds in their headspace with concentrations around 51-71% of the total aroma (as calculated with data of Table 1 and 2), being hexanal the most characteristic compound in the headspace of these non-climacteric lines with concentrations around 51-55% (Table 1 and 2) which allowed to distinguish the non-climacteric lines from the rest (Fig. 3 and 4, Supplementary Fig. 1).

4. Discussion

The present experiment investigates for the first time the aroma profile of the climacteric NILs obtained from non-climacteric parentals (Obando et al., 2007). Aroma compounds associated with climacteric and non-climacteric behavior during melon ripening could be discriminated using reference genotypes, the control genotypes and newly developed NILs with different ripening behaviour in terms of respiration rate and ethylene production.

The carbon compounds and the formation of carbon skeletons are necessary for postharvest maintenance and synthetic reactions to produce the aroma precursors, such as amino acids, nucleotides, pigments, lipids and flavor compounds (Kays and Paull, 2004; Goff and Klee, 2006). A high respiration rate leads to a high breakdown of the carbon

compounds and a shorter shelf life of the commodity. In fact, in this experiment the harvest of the fruit of NIL SC3-5 and others climacteric NILs that contained shorter introgressions in LG III, and fruit from ´Fado´ and ´Védrantais´ started up to 5 to 7 days earlier than fruit from PS, SC, 5M7 or ´Nicolás´. The shelf-life of ´Védrantais´ and ´Fado´ was shorter than that of the climacteric NILs, in agreement with the higher and advanced ~~RR~~ respiration rate and ethylene production~~EP~~ (Fig. 1; Fernández-Trujillo et al., 2008), typical of the climacteric behavior of these cultivars (Flores et al., 2002; Périn et al., 2002; Kourkoutas et al., 2006).

The genetic control of ethylene production in melon is complex. Périn et al. (2002) found that 2 major loci control autocatalytic ethylene production and at least 4 unlinked QTLs control the level of ethylene production. None of these major genes or QTLs were found in the regions of LG III that contain the SC introgressions of SC3-5 and the rest of NILs of this study (Fernández-Trujillo et al., 2008; Moreno et al., ~~unpublished~~in press), indicating that the genetic control of climacteric behavior in the current population could be different and this matched with different aroma profile. Because of SC3-5 was the only NIL from the collection developed by Eduardo et al. (2005) that showed a clear climacteric ripening (Moreno et al., ~~unpublished~~in press), this made us to test only NILs with introgression in LG III. However, it cannot be discarded that additional introgressions from SC may be present in any of the NILs tested (Eduardo et al., 2005). Thus, the climacteric ripening in the recurrent NILs must be controlled by a very small number of QTLs, strongly linked to ethylene-dependent genes in charge of the emission of aroma. Further work is necessary to assess if any of those QTLs correspond to the QTL effects observed in SC3-5.

The introgressions from SC into PS not only provoked changes in the aroma profile,

but also a light yellow to orange flesh in the NILs other than 5M2 or 5M7 (white to very light yellow flesh), and particularly in 5M4 (data not shown). The carotenoids present in the climacteric NILs were probably xantophylls and β-carotene (Watanabe et al. 1991; M.J. Rodrigo, personal communication, 2007). Ibdah et al. (2006) found that carotenoid pigmentation patterns have profound effects on the aroma volatile compositions of tomatoes and watermelon fruit, and those pigments will produce apocarotenoids from their oxidative cleavage, which is important for flavor in diverse food products (Goff and Klee, 2006). Indeed, carotenoids, along with the amino acids valine, isoleucine, methionine and alanine, have been postulated as the precursors of aromatic esters (Wyllie et al., 1995; Wang et al., 1996; Ibdah et al., 2006), the non-acetate esters being the major compounds present in melon SC3-5, the climacteric NILs 5M4, 5M6, 5M9, 5M10 and in the climacteric cultivars Fado and Védrantais, in agreement with results obtained for highly aromatic melons (Lamikanra and Richard, 2002; Beaulieu, 2005; El-Sharkawy et al., 2005; Zhu et al., 2005; Beaulieu, 2006). The compound methyl butyrate, even though not classified as a good discriminant, was not detected in the non-climacteric NIL 5M7 but was present in a higher concentration in the climacteric NILs, PS and the reference cultivars.

The acetate esters identified in the NILs (Table 1) have also been reported in the aroma and fruity character of numerous fruit, particularly in cantaloupe melons (Shalit et al., 2001; Lamikanra and Richard, 2002; Yariv et al., 2004). High concentrations of acetates, such as ethyl acetate, present in higher concentration in the NILs compared with PS, are associated either with a high degree of ripeness (Senesi et al. 2005) or with the fermentation process (Baldwin, 2000). However, this degree is not only ripening-dependent, but also cultivar-dependent, and maybe related to differences in AAT activity between SC and climacteric cultivars such as ´Galia´ or ´Védrantais´ (Shalit et al., 2001;

Yariv et al., 2004; El-Sharkawy.et al., 2005). For example, this compound showed a different behavior in ´Fado´ and in *C. melo* L. reticulatus group cv. Makdimon melons because it was the most abundant volatile produced by the fruit during their final growth stage and during ripening (Wang et al., 1996).

The aldehydes found in the NILs have also been identified in the aroma of climacteric and highly aromatic varieties of melon (Lamikanra and Richard, 2002); for example, acetaldehyde and hexanal have been described as being responsible for the pleasant aroma of cantaloupe melon (Beaulieu, 2006) and high concentrations of these compounds are associated with a very high degree of ripeness in muskmelons (Senesi et al., 2005), probably as a result of the ethylene stimulation of lipoxygenase activity (Baldwin, 2002; Bellincontro et al., 2006).

Ethanol was only higher in 5M4 compared with PS. Senesi et al. (2005) associated a high ethanol concentration with advanced melon ripeness with or without off-flavor. Two sulfur-derived compounds of the profile, which showed noticeable differences between climacteric NILs and PS (Tables 1 and 3), have always been associated with the heating and/or cooking process of food (Mussinan and Keelan, 1994). For example, they have been reported as key contributors to dry-cured ham flavor (García-Esteban, 2004). However, due to the high quantities detected in some melon types such as Cantaloupes they can contribute to musky aroma notes by the action of AAT and determine consumer preference (Wyllie et al., 1995; Ueda et al., 1997; Kourkoutas et al., 2006; Luchetta et al., 2007).

The acetone and camphor, which did not clearly discriminate climacteric from non-climacteric NILs (Table 2; see above section 3.5), have been described as components of the aroma of several varieties of melon (Lamikanra and Richard, 2002). Beaulieu & Grimm (2001) reported that ketones were dominant in immature Cantaloupe melons, but they were

recovered in only trace levels or were not detected in mature fruit.

Though SC showed the same non-climacteric behavior as PS (Fig. 1), its aroma composition was similar to some extent in some individual compounds to ´Fado´ and ´Védrantais´ (Table 1; Supplementary Fig. 1). However, in Fig. 3 the PC2 of the PLS-DA was able to discriminate between SC and the other ´Védrantais´ and ´Fado´. Both cultivars that show a pronounced climacteric behavior (Fig. 1; data not shown for ´Fado´; Flores et al., 2002), showed a clearly distinct aroma from the NILs and their inbred parentals, PS or SC (Supplementary Fig. 1), as it is also shown by the ellipses drawn around each centroid (Supplementary Fig. 2).

Finally, the NILs with less pronounced climacteric behavior (section 3.1) were not clearly discriminated in two groups by using PLS-DA of the aroma profile. In fact, they were located in the positive direction of the latent variable PC1 (Fig. 4) with the exception of 5M10 and particularly 5M6 (in the negative direction). This indicates that different degrees of aroma in the climacteric NILs can be achieved depending on the SC introgressions.

5. Conclusions

The NIL SC3-5 and other 7 NILs with different introgressions on LG III from SC on the PS (non-climacteric) genetic background showed peak levels of respiration rate and ethylene production typical of climacteric ripening. These levels were lower than the standard melon climacteric cultivars ´Fado´ and ´Védrantais´. The analysis of the aroma profile composed by 28 volatile compounds (mainly esters, and, in lower concentration, aldehydes, alcohols and sulfur compounds) ~~showed~~ indicated that the climacteric NILs

showed a volatile profile similar to the standard climacteric cultivars. Three compounds (isobutyl acetate; benzyl acetate; pentanal) allowed the climacteric NILs to be distinguished from the non-climacteric, and their efficacy in this respect was confirmed by the statistical analyses conducted (PLS, ANOVA followed by a Dunnett´s test). However, the profile was not enough to discriminate between the two groups of intensity of climacteric behavior identified. Given the near-isogenic nature of the NILs reported here, the dramatic change in aroma profile should be under a simple genetic control. The fact that the climacteric NILs shared the aroma profile suggests that this new profile is a pleiotropic effect of the climacteric ripening induced by the SC introgressions. Therefore, the results exposed in the current report reinforced the strong genetic relationship between aroma profile and ripening in melon. The NILs derived from SC are an appropriated genetic system to shed light on the relationship of aroma formation and ripening.

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Figure captions

Figure 1. Changes in respiration rate (A, C) and ethylene production (B, D) (mean ± SE, n=5) during postharvest ripening at 21 ºC in the parental lines ´Piel de Sapo´ (PS), the exotic Korean accession ´Shongwan Charmi´ [SC (~~PI161375~~PI 161375)], reference cultivars (hybrid ´Piel de sapo´ type cultivar Nicolás, and *C. melo* var. *cantalupensis* cultivar Védrantais), a near-isogenic line of melon (NIL SC3-5) and two derived NILs (5M3 and 5M9) representative of the two main patterns found in the climacteric NILs and NIL 5M7 with a non-climacteric behavior similar to PS.

Figure 2. Total ion spectra from commercially ripe ´Piel de sapo´ melon (*Cucumis melo*) fruit obtained from the headspace by solid-phase micro-extraction and analyzed by gas chromatography-mass spectrometry. The composite chromatogram of 20 replicates in duplicate is presented. Retention times beside the peaks correspond with Supplementary Table 1.

Figure 3. Plots of centroids after partial least square analysis (PLS) of aroma compounds detected in the headspace of fruit of ´Piel de sapo´ (PS), ´Shongwan Charmi´ [SC (~~PI161375~~PI 161375)], Galia cv. Fado, ¨Nicolás´, and ´Védrantais´. The first and second latent components (PC1 and PC2, respectively) were located on x and y axes. The first and the second latent variables explained 61% and 25% of the X-variance (aroma volatile variables) respectively and 14% and 19% of the Y-variance, respectively. Ellipses represent r^2 = 50 and 100 % explained by the model.

Figure 4. Plots of centroids after partial least square analysis (PLS) of aroma compounds detected in the headspace of fruit of ´Piel de sapo´ (PS) and near-isogenic lines (NILs). The first and second latent variables (PC1 and PC2, respectively) were located on x and y axes. The first and the second latent variable explain 59% and 6% of the X-variance (aroma volatile variables) respectively and 14% and 5% of the Y-variance, respectively. Ellipses represent r^2 = 50 and 100 % explained by the model.