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Article

Differences in the Chemical Composition of Melon (*Cucumis melo* L.) Nectar Explain Flower Gender Preference by Its Pollinator, *Apis mellifera*

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Melon is a pollinator-dependent crop that relies mainly on bees to set fruits. However, man-selected varieties vary in their attractiveness to flower visitors, and some flower genders and melon types may be less visited than others, harming pollination. We investigated the nectar composition of male, female and hermaphrodite flowers of 270 individuals of five commercial melons *Cucumis melo* (Cantaloupe, Charentais, Galia, Piel de Sapo, and Yellow), and its role in flower visit by *Apis mellifera* foragers. We found that melon nectar is composed mainly of sugars and amino acids such as tyrosine, phenylalanine, tryptophan, and flavonoids kaempferol-3-*O*-neohesperidoside, luteolin hexoside, and kaempferol rhamnoside. But the amount of these chemical compounds varies among the flower genders. We also developed an accurate regression model to predict the number of bee visits to melon flowers based on the nectar composition. Our results indicate that nectar composition plays little role in bee discrimination among flowers of different melon types but is essential to the honeybee choice between flower gender. The amounts of phenylalanine (49.40%) and tryptophan (12.05%) in the nectar are related to bee preference for hermaphrodite flowers. More visits to hermaphrodite flowers contribute to setting and developing well-formed fruits, increasing productivity.

Keywords: crop pollination, flower choice, nectar chemometric analysis, phenylalanine, tryptophan

Introduction

The world population is presently estimated at 7.9 billion people and will reach 9.7 billion human beings by 2050.[1] The constant population growth pressures for increments in food and other agricultural products lead to the worldwide expansion of cultivated land over natural areas and increase deforestation and environmental impact risks.[2-4] A potential response to compromise the growing demand for agricultural goods and ecological conservation is increasing agricultural productivity to produce higher yields in the same cultivated areas.[5-7]

Of the many measures already used in agriculture to increase crop yields, such as selection of varieties of higher productivity, better irrigation, soil fertilization, and protection against pests and diseases, pollination seems to be crucial because most cultivated plant species produce better yields (quantitatively or qualitatively) when properly pollinated and none of those agricultural practices have good results at the end if the flowers are not pollinated to set the fruits.[3,8]

Melon (*Cucumis melo* L.) is a crop highly dependent on biotic pollination.[8,9] The crop originated in Africa but is cultivated in 101 countries worldwide for its refreshing

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fruit.[8,10] The species has the highest phenotypic variability in its genus. While the plant and flowers look similar, their morphological diversity is more accentuated in the fruits, where there are variations in the colors, shapes, and sizes.[11,12] To facilitate commercialization, the cultivated melons were grouped into a so-called "type" classification, composed of a group of cultivars with similar characteristics, easily identified and differentiated by the appearance of the fruit skin (color, presence or absence of sutures, scars, reticulation or tracing), fruit shape and pulp color.[13,14]

Melon is the third most cultivated cucurbit globally, reaching a global production of 28.4 million tons in 2020 from a harvested area of 1.07 million hectares.[15] China (48.7%), Turkey (6.0%), India (4.67%), Iran (4.51%) and Kazakhstan (4.10%) are the world's largest melon producers and represent 67.98% of the global melon production. In 2020, the average worldwide yield for the melon crop was 26.4 tonnes *per* hectare. Still, countries like China and the USA had an average melon crop yield of over 35.0 tonnes *per* hectare nationwide, showing there is room for yield improvement in this crop.[15] Yield increments through adequate pollination could prevent new areas from being deforested and incorporated for melon cropping.

Melon pollination, however, is tricky. While most cultivated types bear male and hermaphrodite flowers, a few types also produce female flowers. Although all types of melon and flower gender are self-compatible, all flowers last only one day, and those capable of bearing fruits, the hermaphrodite and female flowers, are produced in much lower numbers than male flowers (average proportion 1:6-19).[16-20] Also, hermaphrodite and female flowers have hundreds of ovules in their ovaries. To set fruit and for fruits to develop to commercial size and shape, an average of 400 to 600 of these ovules have to be fertilized.[21,22] Therefore, many viable pollen grains have to be transferred from stamens to receptive stigmas within a few hours of the flower's opening. This means a high pollen transfer rate that can only be achieved in most hermaphrodite/female flowers of a cultivated area if pollinators are present in adequate numbers and frequently moves between flowers of distinct gender, especially from male to hermaphrodite and female flowers.[23,24]

Recent studies[20,25] have shown that honeybees (*Apis mellifera*), the main pollinator species used for melon pollination worldwide, can discriminate among flowers of different agronomic types of melon and indicate a preference for visiting some of them over others. In such a situation, areas grown with the less favored melon types may not be well pollinated, and larger areas need to be cropped to compensate for the losses in yield.

Honeybee recognition and preference among flowers of different melon types are possible because each melon type produces a unique blend of attractive and repellent volatile organic compounds (VOCs) easily distinguished by the bees.[25] However, honey bees also discriminate between male, hermaphrodite, and female flowers of melon, but VOCs seem to play little role in their choice among flower genders.[9,20] Understanding what factors determine the bee foraging behavior and flower preference is important because more visits to hermaphrodite and female flowers are desirable to ensure efficient pollination, high yields, and better quality fruits.

Considering that honeybees visit melon flowers for pollen and nectar, but nectar is the only resource available all day long in the three flower genders, we believe that melon nectar may play a decisive role in flower gender preference by the honeybees.

Given this fact, an easy and effective method for the simultaneous and rapid detection of the composition of sugars, polyphenols and amino acids in the corresponding nectars of five melon species of three different genders was developed. It was established by ultra-performance liquid chromatography coupled with high resolution mass spectrometry (UPLC-HRMS). Complementarily, multivariate statistical tools such as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were applied to assess their ecological relationships.

Therefore, in this study, we investigated the nectar composition of melon flowers, aiming to (*i*) identify, quantify and qualify the nectar compounds of melon flowers; (*ii*) investigate differences in the profile of chemical compounds produced according to the flower gender and agronomic types of melon; (*iii*) investigate possible relationships between chemical compounds present in the nectar produced by the melon flowers and the observed number of floral visits by *Apis mellifera* foragers.

Experimental

Reagents

Formic acid LiChropur™ (98-100% high-performance liquid chromatography (HPLC), Merck, Germany), acetonitrile and methanol Chromasolv™ (≥ 99.9% liquid chromatography mass spectrometry (LC-MS), Honeywell, Germany) were the solvents for running the samples. Leucine-enkephalin (Waters Technologies, USA) were used as external control standards. Reference standards were applied to compare retention time, MS^1 and MS^2 data including, quercetin-3-*O*-rhamnoside (≥ 95% HPLC,

Sigma-Aldrich, USA) was used as an external control standard analyzed and quercetin (≥ 95% HPLC, Sigma-Aldrich, USA). All aqueous solutions were prepared using a Milli-Q™ system-producing ultrapure water (18.2 MΩ cm^{-1}; Millipore, USA).

Field experimental design

The field experiment was carried out during the dry seasons in November 2014 and November 2015 at the Experimental Field of the Brazilian Agricultural Research Corporation (Embrapa), located in the municipality of Pacajus, state of Ceará, NE, Brazil. In both years, five commercial melon types were cultivated in an area of 800 m^2 made of 20 rows, each row split in two halves, totaling 40 plots. Each type of melon was grown in eight replicate plots in an entirely randomized design, and each plot comprised 25 melon plants. Melon types were Yellow, Cantaloupe, Piel de Sapo, Charentais, and Galia, all represented by a hybrid of good commercial acceptance; Goldex, Zelda, Ricura, Banzai, and McLaren, respectively. The crop was grown following all agronomic techniques for cultivating melons, but pesticides were avoided because they could affect bee visitation to the flowers.

After the melon plants started flowering, we moved two strong and healthy colonies of the Africanized honeybee, *Apis mellifera*, into the experimental area to provide floral visitors. More details regarding crop experimental design, melon cultivation, honeybee introduction and management, and data collection on *A. mellifera* discrimination between melon flowers and differentiated visitation rates to the distinct agronomic types of melon are given in Fernandes.[25]

Nectar sampling

Before nectar sampling, we used muslin bags to isolate as many pre-anthesis flower buds from male, hermaphrodite, and female flowers of all melon types as possible to prevent visitors by bees and other floral visitors. The following day, when flowers were already open, we sampled nectar using a 5 µL capillary at two-hour intervals from 7 a.m. to 5 p.m., when night began to fall in the studied area.[26] After each sampling, the flower was discarded. The nectar sampled was transferred to a vial marked according to the flower gender and melon type, which was kept refrigerated at 3 °C until the next sampling two hours later. Then, the nectar sampled from another flower of the same gender and melon type was mixed with the previous vial samples. Thus, by 5 p.m., each vial contained a mixture of the nectar sampled from a specific flower gender and melon type six times throughout the day. Three replicates of these composite samples were taken to each flower gender and melon type *per* day, and we sampled the crop for three alternate days (6 × 3 × 3 = 54 individuals *per* flower gender). Considering that three melon types presented two flower genders (54 × 2 = 108), while two melon types presented three flower genders (54 × 3 = 162), a total of 270 flowers of different individuals were sampled. Due to the large number of samples and in some cases, a small volume of nectar *per* flower collection, it was decided to group the samples so that the concentration of metabolites reached the detection and sensitivity range of the equipment. Therefore, the 270 collections gave rise to pools totaling 66 samples.

UPLC-QTOF-MS^E (ultra performance liquid chromatography-high-resolution mass spectrometry, Xevo QTof MS^E) analysis was performed to evaluate the variability of nectar composition according to five commercial types of melon (Yellow, Cantaloupe, Charentais, Galia and Piel de Sapo) and three genera of melon flowers (male, female, and hermaphrodite). The 66 nectar samples resulted from a duplicate of the sampling of the year (biological replication) and a triplicate of the chromatogram acquisition (66 × 2 × 3 = 396 chromatograms). At the end of each day, the composite samples were taken to the Natural Products Laboratory of the Brazilian Agricultural Research Corporation (Embrapa) in Fortaleza and frozen at –80 °C until analysis.

Sample preparation

Each nectar pool sample (50 µL) was dissolved in 1 mL of methanol:water solution (50:50 v/v) with the function of precipitating the proteins present in the sample. The mixture was shaken with a vortex mixer for 1 min and then centrifuged for 5 min at 3,000 rpm (1,008 g) in a Sorvall™ Legend™ X1R centrifuge (Rotor 75003623; Thermo Fisher Scientific, USA). After the end of the centrifugation process, an aliquot of 500 µL of the supernatant was removed, filtered in a with Millex-HV Durapore™ Acrodisc Filter (13 mm × 0.22 µm) Syringes (SLHVX13NL, PVDF (polyvinylidene difluoride); Merck Millipore, Germany) and stored in a 2 mL vial for further injection and analysis via UPLC-HRMS.

UPLC-HRMS analysis

The main compounds present in the nectar of melon flowers were annotated using UPLC-QTOF-MS^E according to their retention time, and mass spectra by comparison with data from the literature. The chromatographic separation was performed using a Waters Acquity UPLC BEH (150 × 2.1 mm × 1.7 µm) column under a temperature

set at 40 °C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Chromatographic separation was accomplished using a gradient of 2-95% of B (0-15 min), 100% B (15.1-16.0 min) and reconditioned with 2% B (16.1-19.1 min) in a flow of 0.4 mL min^{-1} and injection volume of 5.0 µL *per* sample.

A Xevo™ QTof-MS mass spectrometer (Waters Corporation, USA) equipped with a ZSpray™ source operating in positive electrospray ionization (ESI^+) was used for analyses. N_2 was used as the desolvation gas produced by an NM30LA-MS high-purity N_2 generator (Peak Scientific Instrument™, Scotland), which was used as the cone gas, desolvation temperature, and flow at 350 °C and 500 L h^{-1}, respectively. Argon ≥ 99.9% purity (White Martins, Brazil) was used as a collision gas at a pressure of 1.4×10^{-2} mbar. The instrument was calibrated using a sodium formate standard solution and MassLynx™ 4.1 software (Waters Corporation, USA) was used for data acquisition.

Mass accuracy and reproducibility were ensured by leucine-enkephalin infusion into the lock mass system (400 ng mL^{-1}), $[M + H]^+$ ion *m/z* 556.2771, and every 20 s on the LockSpray flow probe at 20 µL min^{-1}. Quercetin-3-*O*-rhamnoside was used as an external control standard and analyzed every 10 injections for stability data of UPLC-HRMS during the experiment.

The ESI^+ mode was acquired in the range of 110-1180 Da in MS^1 and in the range of 50-1180 Da in MS^2. The spectrometer operated with MS^E centroid programming using a tension ramp from 20 to 40 V.

Since in a mass spectrometry analysis the compounds may preferentially ionize in positive mode, such as flavonoids and alkaloids, or negative mode as phenolic and carboxylic derivatives, the UPLC-HRMS analysis was performed using both ionization modes. Consequently, the positive ionization mode was chosen for the development of this study according to the relevance of the results (data available in Supplementary Information (SI) section).

Peak annotation

The data set was imported to Mass Spectrometry-Data Independent Analysis software (MS-DIAL 4.16),[27] aimed at implementing functions required for untargeted metabolomics, such as obtaining deconvoluted spectra, peak alignment, and filtering. Thus, MS-DIAL is a prerequisite for compound identification. Posteriorly, the unknown metabolites can be identified by their elemental formulas and *in silico* mass spectral fragmentation with MS-FINDER 3.40.[28] Structural annotations were based on molecular formula and MS/MS fragmentation with activated heuristic rules. After obtaining the MS/MS spectra, the annotation of the compounds was performed, comparing the data with the information from the database such as the ChemSpider,[29] SciFinder,[30] and PubChem.[31] Following the metabolic standards initiative (MSI) level 2.1 parameters, a putative annotation was obtained, including molecular formula and MS^E fragments. In addition, it is important to mention that chemical identification was based on chemotaxonomy (family, genus, and species).

Chemometric analyses

Chemometric analysis was performed to evaluate the variability of the nectar composition according to five commercial types of melon (Yellow, Cantaloupe, Charentais, Galia, and Piel de Sapo) and three genders of melon flowers (male, female, and hermaphrodite), which resulted in 66 nectar samples from a duplicate of the year sampling (biologic replicate) and triplicate of chromatograms acquisition. The samples were named according to the melon type (Yellow (YE), Cantaloupe (CA), Charentais (CH), Galia (GA), and Piel de Sapo (PS)) and flower gender (male (M), female (F), or hermaphrodite (H)).

Initially, an untargeted chemometric analysis by principal component analysis (PCA) was developed considering the chromatograms region between 0.5 and 6.0 min, which resulted in a numerical matrix with a dimensionality of 238,392 points (602 variables *per* chromatogram × 396 chromatograms). For this, the chromatograms data were converted to American Standard Code for Information Interchange (ASCII) files, imported by Origin™ 9.4[32] program to numerical matrix construction, and exported for chemometric analysis using The Unscrambler™ X (version 10.4)[33] program. Algorithms for baseline correction and mean centering were applied for dataset pre-treatment, and the SVD (singular value decomposition) algorithm was used to decompose the numerical matrix in scores, loadings and residual data. The use of this method made possible a clear understanding of the correlation between the chemical profile of the nectar with melon type and flower gender.

Additionality, a targeted PCA was developed considering the absolute area of the non-overlapping signals from the identified compounds that were highlighted by the first PCA (described in the previous paragraph). The data was autoscaled (mean-centered divided by standard deviation), and the SVD algorithm was used to decompose the numerical matrix in scores, loadings and residual data.

A supervised chemometric modeling by partial least squares discriminant analysis (PLS-DA) was applied to

classify the chemical variability of the nectar according to the flower gender and/or melon type, which was performed by the NIPALS (nonlinear iterative partial least squares) algorithm with the number of latent variables (LV) selected by the following statistical parameters:[34-36] total variance refers to three LV (LV1 + LV2 + LV3); root mean square error of calibration (RMSEC); coefficient of correlation between the real and predicted bees visit during the calibration (R^2_{cal}); root mean square error of cross-validation (RMSECV); coefficient of correlation between the real and predicted bees visitation during the validation (r^2_{val}); root mean square error of prediction (RMSEP); coefficient of correlation between the real and predicted bees visitation during the prediction (R^2_{pred}). Furthermore, a supervised PLS model was constructed using the number of bee visits as independent variables (Y column) to detect possible marker compounds in nectar regarding the flower gender and/or melon type, excepting the female flowers by the absence of visit data. All the multivariate modeling was performed using the complete cross-validation method under 95% of confidence level: each chromatogram was left out from the calibration dataset, and the models were calibrated on the remaining data points.

Results

Chemical profile by UPLC-QTOF-MS^E and chemometric analysis

Nectar samples from the five melon types (Yellow, Cantaloupe, Charentais, Galia, and Piel de Sapo) and three genders of the melon flowers (male, female, and hermaphrodite) resulted in 396 chromatograms, which are shown in Figure 1.

The main organic compounds identified in the nectar, using UPLC-HRMS acquisition (data available in SI section), and some classes found are sugars such as sucrose isomers, amino acids such as tyrosine, phenylalanine, tryptophan, and flavonoids kaempferol-3-*O*-neohesperidoside, luteolin hexoside, and kaempferol rhamnoside (Figure 1).

The scores mean of the nectars from the five melon types and three flower genders considering the replicate of sampling over two years (2014 and 2015) and triplicate of UPLC-HRMS acquisition, with loadings represented by vectors from the origin, are shown in Figure 2. Significant composition tendencies were observed based on the first two principal components (PC1 and PC2), representing 85.0% of the total variance.

In general, regardless of the type of melon, nectars from male flowers were located at negative scores of PC1, and nectars from hermaphrodite flowers were positioned

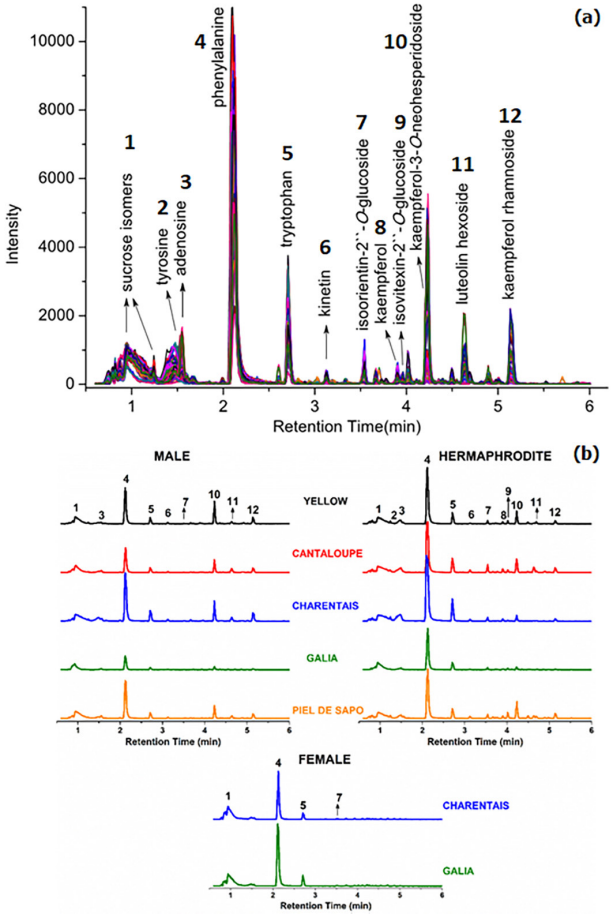

Figure 1. Overlay of chromatograms acquired on UPLC-HRMS of all types and genera of melon (a). In part (b) we have the representation of the general chromatographic profiles stacked separately for each type and gender of melon where nectar was collected from male, female and hermaphrodite flowers of five commercial types of melon acquired in UPLC-HRMS in positive ionization mode.

at positive scores of the same PC. Nectars from female flowers were situated at the most positive scores of PC2. According to the PC1 loadings, male flowers influenced the highest amounts of adenosine (1.55 min), kaempferol-3-*O*-neohesperidoside (4.23 min), luteolin hexoside (4.64 min), and kaempferol rhamnoside (5.13 min) in nectar. On the other hand, the hermaphrodite flowers produced nectars with the highest amounts of phenylalanine (2.09 min), tryptophan (2.71 min), and isoorientin-2'-*O*-glucoside (3.55 min). According to the PC2 axis, all-female flowers influenced the lower production of the compounds mentioned above.

The robustness of the clustering in the PCA model and the capacity to classify nectars according to the type of melon and/or gender of the flower were verified by PLS-DA modeling (scores and loadings available in SI section), which were constructed separately for each parameter (flower gender and melon type). The classification model for the variability of nectar according to the gender of the

Table 1. Statistical parameters from the multivariate classification models regarding the gender of the flower and type of melon and regression model for bee visitation using 3 LV

Model	3 LV[a] / %	RMSEC[b]	r^2cal[c]	RMSECV[d]	r^2val[e]	RMSEP[f]	r^2pred[g]
Flower gender	82	0.304	0.82	0.647	0.32	–	–
Melon type	21	1.170	0.21	2.693	0.00	–	–
Bee visitation	96	1.888	0.96	4.260	0.82	1.888	0.96

[a]Total variance refers to three latent variables (LV1 + LV2 + LV3); [b]root mean square error of calibration; [c]coefficient of correlation between the real and predicted bees visit during the calibration; [d]root mean square error of cross-validation; [e]coefficient of correlation between the real and predicted bees visitation during the validation; [f]root mean square error of prediction; [g]coefficient of correlation between the real and predicted bees visitation during the prediction.

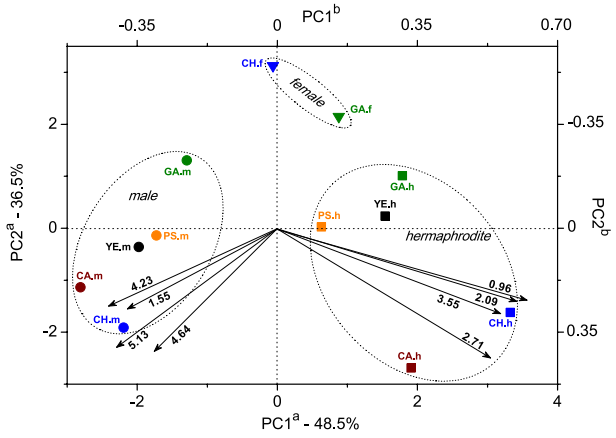

Figure 2. PCA biplot of nectar from YE: Yellow (black), CA: Cantaloupe (red), CH: Charentais (blue), GA: Galia (green), and PS: Piel de sapo (orange) melons, considering the flower gender: male in circles, female in triangles, and hermaphrodite in squares. The axes referred to scores are overwritten by letter "a" with percentage values of the explained variance on each principal component, and the axes overwritten by letter "a" are referred to loadings represented by vectors from the origin: 0.96 min: sucrose isomers; 1.55: adenosine; 2.09: phenylalanine; 2.71: tryptophan; 3.55: isoorientin-2'-*O*-glucoside; 4.23: kaempferol-3-*O*-neohesperidoside; 4.6: luteolin hexoside; 5.13: kaempferol rhamnoside.

flower was better adjusted than the model according to the type of melon.

Additionally to classification, a multivariate regression model by PLS was constructed using the number of bee visits in melon flowers as an independent variable (Y column) to detect possible marker compounds related to the flower gender and/or melon type. The chosen discriminant factor (bee visitation) is of paramount importance because the bees are responsible for pollination, yield, and quality of melon fruits.[22,24,37] The reliability and robustness of all the models are represented by the statistical parameters presented in Table 1: RMSEC; RMSECV; RMSEP; respective correlation coefficients (r^2).

Based on the regression model, we compared the real and predicted number of bee visits in male and hermaphrodite melon flowers, as shown in Table 2 (scores and loadings available in SI section). According to the relationship between the nectar composition and the number of bee visits, the model predicted the number of visitors for female flowers with elevated prediction ability (r^2 = 0.96) using 3 LV (Table 2). Female flowers were produced in small numbers, appearing four to five days after the first male flowers and only by Charentais and Galia out of the five melon types studied.[20] They were the least attractive and visited flowers by the bees, and probably their role in melon yield is not relevant.

Table 2. The real number of bee visits to male (M) and hermaphrodite (H) flowers from different types of melons and the predicted bees visitation based on the prediction model, with the regression coefficient (r^2) of 0.96 and the respective standard deviation

Melon type (flower gender)	Real visitation	Predicted visitation (r^2 = 0.96)	Deviation[a]
YE (M)	37[a]	39[a]	4.0
YE (H)	49[b]	47[b]	2.3
CA (M)	46[c]	43[c]	3.6
CA (H)	61[a]	61[a]	2.9
CH (M)	30[a]	31[a]	3.2
CH (H)	43[a]	43[a]	3.1
GA (M)	32[a]	34[a]	2.7
GA (H)	45[a]	43[a]	3.2
PS (M)	38[a]	36[a]	2.9
PS (H)	53[a]	56[a]	2.8

[a]Express the uncertainty of the prediction estimated as a function of the global model error, samples leverage, and the residuals X-variance. Same overwritten letters in the lines represent the statistical equality between the values used to develop the model and from prediction. YE: Yellow; CA: Cantaloupe; CH: Charentais; GA: Galia; PS: Piel de Sapo.

The classification models of nectars based on flower gender and melon type presented the first latent variable (LV1) as the main axis responsible for the placement of the sample. The loadings corroborated the strong relationship between the hermaphrodite flowers and the high amounts of phenylalanine, tryptophan, and isoorientin-2''-*O*-glucoside in nectars as higher amounts of adenosine, kaempferol-3-*O*-neohesperidoside, luteolin hexoside, and kaempferol rhamnoside in nectars from male flowers. The respective scores and loadings are made available in SI section. The model based on the type of melon was not well-adjusted (data not shown), suggesting that nectar composition plays

a minor role in honeybee discrimination among melon types. However, in general, hermaphrodite flowers with positive scores were more attractive to pollinators than the male flowers.

The multivariate classification and regression models indicated that the compounds phenylalanine and tryptophan in the nectar are directly related to bee attraction. Furthermore, the model has related male flowers to the highest amounts of adenosine, kaempferol-3-*O*-neohesperidoside, luteolin hexoside, and kaempferol rhamnoside in nectar.

The compounds that presented significant variations according to the melon type and flower gender and did not exhibit overlapping signals in the chromatograms were semi-quantified (expressed as relative concentration). The signals were integrated and based on analysis of variance (ANOVA) single factor (with a significance level of 0.05, using the Tukey's test and Levene to test the homogeneity of variance). Figure 3 presents the relative concentration of the compounds in male (blue) and hermaphrodite (green) flowers, with a standard deviation of the biologic replicates of sampling (years 2014 and 2015) and triplicate of chromatograms acquisition. As noted in chemometric evaluation, the nectar of male flowers contains higher amounts of adenosine, kaempferol-3-*O*-neohesperidoside (KaeHes), and luteolin hexoside (LutHex), and kaempferol rhamnoside (KaeRha) than that of hermaphrodite flowers. On the other hand, the nectar of hermaphrodite flowers presented the highest amounts of phenylalanine and tryptophan (Table 2). In particular, hermaphrodite flowers from Charentais (CH) melon type produced the highest amounts of phenylalanine and tryptophan, followed by Cantaloupe (CA), which was the most visited flower.

Discussion

Chemical profile by UPLC-QTOF-MS^E and chemometric analysis

The perfect match of the 396 chromatograms resulting from the five melon types (Yellow, Cantaloupe, Charentais, Galia, and Piel de Sapo) and three genders of the melon flowers (male, female, and hermaphrodite) suggest that melon nectar is composed of the same main chemical compounds regardless of flower gender and melon type. Also, the leading organic compounds found in the melon nectar, carbohydrates, and amino acids, are components of floral nectars of many plant species[38-42] and these classes of compounds play an essential role in the ecological plant-insect interactions, serving as an attractant and nutritional components to nectarivores flower visitors.

However, the score means of the nectars from the melon types and flower genders of UPLC-HRMS acquisition showed that although these nectars are composed mainly of the same compounds, they become distinct among flower genders due to male, hermaphrodite and female flowers present significant differences in the amounts of some of these compounds in their nectars. Honeybees can distinguish variations in the composition and concentration

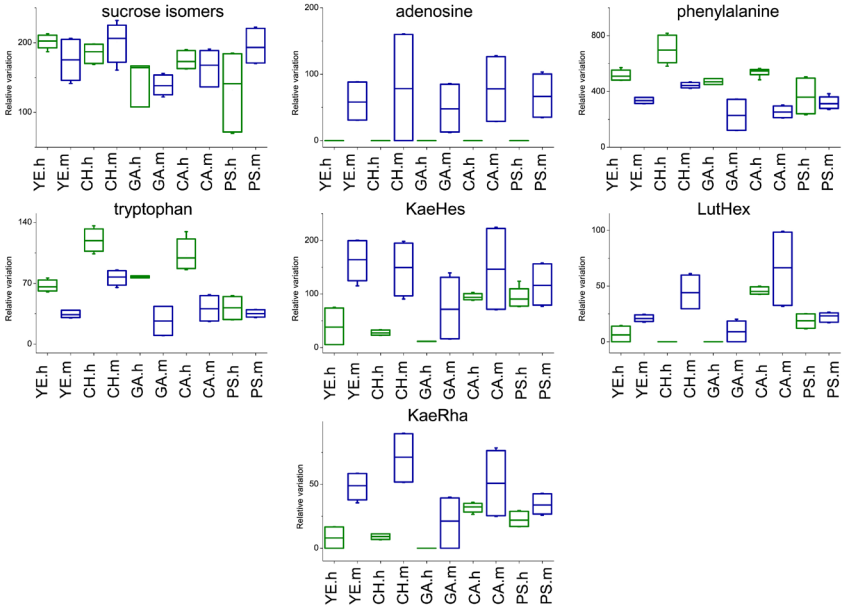

Figure 3. Relative concentrations of the organic compounds in the chromatograms according to the melon type (YE: Yellow, CH: Charentais, GA: Galia, CA: Cantaloupe, and PS: Piel de sapo) and flower gender (hermaphrodite in green, and male in blue color). KaeHes: kaempferol-3-*O*-neohesperidoside; LutHex: luteolin hexoside; KaeRha: kaempferol rhamnoside.

of compounds in nectar,[43-46] and in the case of melons may use this ability to discriminate between flower genders.

Also, the classification model for the variability of nectar according to the gender of the flower was better adjusted than the model according to the type of melon, suggesting nectar composition plays a minor role in honeybee discrimination among melon types. This is undoubtedly due to the significant differences among nectar compositions within the same type of melon based on the flower gender, which negatively affected classification. Among flower genders, hermaphrodite flowers at positive scores, in general, were more attractive to pollinators in comparison to the male flowers. Previous studies[18,23] counting the number of honeybee visits to melon flowers have reported their preference for visiting hermaphrodite flowers. Therefore, differences in nectar among flower gender seem to be an evolutionary trait in melons probably associated with the pollination process, while cultivated melon types result from man-driven selection of desired agricultural characteristics, which usually do not consider pollination.[25,47,48]

Pollen is the source of almost all macro and micronutrients important for the proper development and reproductive success of bees,[49] and as it is demonstrated here for the melon nectar, the nutrient content of pollen grains may differ between different species but also between individuals of the same plant species.[50] Therefore, bees need to assess the nutritional content of pollen to ensure adequate nutrient intake for themselves and their young. Ruedenauer *et al*.[51] showed that bees are able to differentiate between different stimuli, for example, they can perceive differences in concentration of a specific nutrient or group of nutrients. The assessment of pollen nutritional quality can be done directly through the perception of pollen nutrients in flowers or indirectly through physiological feedback.[52] Bees use chemo-tactile cues to differentiate pollen from different plant species, indicating that they can also use chemo-tactile cues to detect variations in nutrient composition.[53] Therefore, the ability to perceive differences in the quantity and concentration of nutrients in pollen is used to assess the nutritional quality of different pollen sources, and our study suggests bees use the same strategy when foraging for nectar, indicating that the quantity and quality of available floral resources has a great impact on the attractiveness of flowers.[54]

Considering nectar compounds, the multivariate classification and regression models showed that phenylalanine and tryptophan in the melon nectar are directly related to bee attraction. This information was corroborated by previous studies showing that although sugar concentration in nectar is approximately 100 to 1000 times higher than amino acids, the amino acids significantly affect the attractiveness of nectars.[55-57] Similarly, ants and other pollinators are more attracted by amino acid-rich nectars.[58-60] On the other hand, male flowers contain higher amounts of adenosine, kaempferol-3-*O*-neohesperidoside (KaeHes), luteolin hexoside (LutHex), and kaempferol rhamnoside (KaeRha) than that of hermaphrodite flowers. Therefore, the statistical data indicated that the model could classify unknown nectars (such as those from female flowers) or predict the number of bee visits to melon flowers based on the nectar composition.

The semi-quantification of the compounds that present significant variations according to the melon type and flower gender showed that the nectar of hermaphrodite flowers presented the highest amounts of phenylalanine and tryptophan, which were the most visited flower gender (Table 2). Hermaphrodite flowers are produced in much smaller numbers than male flowers, usually in a proportion 1:6.[16-18,20] Should the melon flower genders produce the same nectar, one would expect bee visits to hermaphrodite flowers to follow the same proportion above instead of biased in favor of the hermaphrodite flowers. Once the bees are visiting flowers of an area cultivated with a specific melon type, the more significant amount of male flowers is essential in keeping the bees attracted to forage in that crop and in providing the great amount of pollen needed to set fruits while a favored nectar composition induce bees to search for hermaphrodite flowers improving pollination and increasing fruit yield.

The classification model for the variability of nectar according to the type of melon showed low adjustment due to high similarity among the nectars they produce, indicating that the honeybee does not use nectar composition to discriminate among melon types. Instead, Fernandes[25] demonstrated that honeybees use the distinct profile of volatile organic compounds (VOCs) to select the melon type to visit.

Therefore, as honeybees approach a melon field, they use the VOCs emitted by flowers to recognize their favored melon type. In contrast, the choice between flower gender is driven by nectar composition as they probe the flowers. Cultivating melon varieties with nectar richer in phenylalanine and tryptophan, especially in melon types which are less favored by the bees, may produce more visits to hermaphrodite flowers, ensuring deposition of the large number of pollen grains needed to set and develop well-formed fruits, increasing productivity and reducing the environmental impact of this crop.

Conclusions

Based on our findings, we can conclude that melon flowers produce a different type of nectar in each flower

gender by changing the amounts of certain compounds in the nectar of male, hermaphrodite, and female flowers. Also, honeybees are able to discriminate among these nectars. They prefer to collect the one richer in phenylalanine and tryptophan, explaining, at least in part, the greater number of visits to hermaphrodite flowers. Such differences are so remarkable that it is possible to predict the number of bee visits to melon flowers based on the nectar composition.

Also, the high similarity among the nectars produced by different types of melon impairs honeybees from discriminating among them, reinforcing the findings of Fernandes[25] that bee discrimination among flowers of different melon types is determined by the floral volatile organic compounds, while demonstrating that the choice between flower gender is driven by nectar composition. Both knowledge are of great importance for the management of bees for melon pollination because it allows adjusting the number of honey bee colonies (population of pollinators) suitable to obtain the best pollination rates, because it is possible to take in account the attractiveness of each type of melon and the flower density of each gender, especially because the latter depends on both the type of melon grown and the plant density (flowers/area) used.

These findings allow drawing win-win strategies to increase visits to hermaphrodite flowers, ensuring better pollination and higher productivity, preventing the expansion of the cultivated area, and reducing environmental impacts.

Supplementary Information

Supplementary information (table of tentative identification in nectars from male, female, and hermaphrodite flowers from different types of melon, figure of the scores and loadings graphs from the exploratory PCA evaluation applied over the whole numerical nectar data, figure of scores and loadings graphs from the classification model by PLS-DA according to the gender of the flowers and figure of scores and loadings from the regression model according to the number of the visitation of bees in male and hermaphrodite flowers) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

N. S. F. was responsible for project administration, conceptualization, analysis, investigation, methodology and writing original draft; L. R. L. for the chemical characterization and chemical analysis; E. G. A. F. for chemometric analysis, analyzed the experimental results, revised the manuscript, writing-review and editing; F. A. S. A. for supervise and conceptualization experiments; G. J. Z. for project administration, analyzed the experimental results, revised the manuscript, writing-review and editing; B. M. F. for project administration, analyzed the experimental results, revised the manuscript, writing-review and editing.

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