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Phenotypic diversity and biometric characterization of seeds among sweet lemon accessions

Diversidad fenotípica y caracterización biométrica de semillas entre accesiones de limón dulce

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ABSTRACT

The sweet lemon originated from a natural cross with the "Sicilian" lemon, which was discovered in an orchard and subsequently propagated from this tree. The biometric characterization of seeds can support the selection of higher-quality material; however, there is no information regarding the biometric characterization or phenotypic diversity of sweet lemon seeds. This study aimed to evaluate the phenotypic diversity and biometric characterization of seeds from nine sweet lemon accessions. A completely randomized experimental design was adopted, with 25 fruits from each of the nine accessions. The seeds were collected from ripe fruits of sweet lemon accessions and evaluated for the number of seeds per fruit, as well as for seed width, thickness, and length. The data were analyzed using descriptive statistics, correlation analysis, and Tocher clustering. The biometric characterization of seeds revealed significant variability in the number of seeds per fruit, with an average of 16.51 seeds per fruit and values ranging from 6 to 29 seeds per fruit. Seed width showed significant differences among accessions, with accession 4 presenting the lowest values. Phenotypic diversity was evaluated using Tocher's optimization algorithm based on a similarity measure. The accessions showed phenotypic variability among them. Two accessions were individually grouped by Tocher's method and were the most divergent, indicating that they may represent useful materials for future evaluation in breeding programs.

Key words: citrus germplasm; morphological descriptors; phenotypic variation; seed biometry; Tocher clustering

RESUMEN

Entre los cultivos frutales, la fresa (*Fragaria × ananassa* Duch.) es una de las especies de mayor imporEl limón dulce se originó a partir de un cruce natural con el limón siciliano, descubierto en un huerto y posteriormente propagado a partir de este árbol. La caracterización biométrica de las semillas puede apoyar la selección de material de mayor calidad; sin embargo, no existe información sobre la caracterización biométrica o la diversidad fenotípica de las semillas de limón dulce. Este estudio tuvo como objetivo evaluar la diversidad fenotípica y la caracterización biométrica de semillas de nueve accesiones de limón dulce. Se adoptó un diseño experimental completamente al azar, con 25 frutos de cada una de las nueve accesiones. Las semillas se recolectaron de frutos maduros de las accesiones de limón dulce y

se evaluaron en cuanto al número de semillas por fruto, así como al ancho, el grosor y la longitud de las semillas. Los datos se analizaron mediante estadística descriptiva, análisis de correlación y agrupamiento de Tocher. La caracterización biométrica de las semillas reveló una variabilidad significativa en el número de semillas por fruto, con un promedio de 16,51 semillas por fruto y valores que oscilaron entre 6 y 29 semillas por fruto. El ancho de la semilla mostró diferencias significativas entre las accesiones, siendo la accesión 4 la que presentó los valores más bajos. La diversidad fenotípica se evaluó mediante el algoritmo de optimización de Tocher, basado en una medida de similitud. Las accesiones mostraron variabilidad fenotípica entre sí. Dos accesiones fueron agrupadas individualmente por el método de Tocher y resultaron ser las más divergentes, lo que indica que podrían representar materiales útiles para futuras evaluaciones en programas de mejoramiento genético.

Palabras clave: agrupamiento de Tocher; biometría de semillas; descriptores morfológicos; germoplasma de cítricos; variación fenotípica

INTRODUCTION

Lemon is a citrus species that includes varieties of high economic value that can be cultivated under various climatic conditions (Subaşı *et al.*, 2024). The sweet lemon (*Citrus limetta* Risso) is a plant native to Asia that originated from natural hybridization involving lemon (*Citrus limon*). The sweet lemon tree stands out for its botanical characteristics, such as larger and more elongated fruits compared with other lemon and acid lime varieties, with a thick peel, yellow coloration, and a slightly rough surface. Lemons and limes are classified as true lemons and can be divided into two distinct natural groups: the first includes common or high-acidity lemons, whereas the second comprises sweet lemons, characterized by low acidity. This variety is used both for the production of oil and flour and in domestic and culinary applications (EMBRAPA, 2003; Kobori & Jorge, 2003).

It is important to highlight that the term “lemon” encompasses a variety of citrus fruits with acidic juice, including true lemons (*Citrus limon*), such as the Sicilian lemon, as well as acid limes, such as Tahiti lime (*Citrus latifolia*) and Key lime (*Citrus aurantifolia*). For simplicity, this study by the Brazilian Institute of Geography and Statistics (IBGE, 2023) will group acid limes with true lemons.

Global lemon production was estimated at 21 million tons, with Asia and the Americas being the main producing regions. Asia accounted for 43.7% of production, while the Americas accounted for 39.5%. The six largest global producers are India, Mexico, China, Argentina, Brazil, and Turkey, which together account for 58.3% of total production. Individual shares in 2022 were as follows: India, 17.5%; Mexico, 14.4%; China, 12.1%; Argentina, 8.4%; Brazil, 7.5%; and Turkey, 6.1% (FAO, 2023; USDA FAS, 2022; FAOSTAT, 2023).

Sweet lemon was discovered by chance from a plant that produced less acidic fruits than other lemon trees, initiating the multiplication of this species (Colecio-Juárez *et al.*, 2012). Despite its commercial importance in the juice processing market worldwide, there are still few studies and limited information about this species. Seed biometric characterization can provide important information for the conservation and utilization of sweet lemon germplasm resources, enabling the efficient and sustainable use of this species. Historically, morphological analyses have been widely used to study variation among citrus species and varieties. These evaluations allow the characterization of phenotypic variation among populations and accessions, contributing to the understanding of ecological and agronomic aspects such as dispersal mechanisms, adaptation, and plant establishment processes (Macedo *et al*., 2009).

The physical characterization of fruits and seeds is of great importance for assessing phenotypic diversity within a species, supporting breeding programs, and evaluating

its relationship with environmental factors (Nascimento *et al*., 2014; Silva *et al.*, 2017; Silveira *et al.*, 2019). Although variability among accessions can be evaluated through agronomic, morphological, and molecular traits (Blind *et al*., 2020), studies based exclusively on biometric descriptors provide an assessment of phenotypic diversity and variability among accessions. In this context, biometric analysis is an important tool for detecting patterns of variation within populations and their relationship with environmental factors (Carvalho *et al*., 2003).

Furthermore, propagation in citriculture is mainly carried out through grafting, which requires the use of seeds for rootstock production (Duarte *et al.*, 2013; Martins *et al.*, 2007). According to Tuzcu *et al.* (1981), rootstocks must be compatible with the scion variety, show resistance or tolerance to major diseases, contribute to high yields and fruit quality, and promote early production and postharvest fruit conservation. The 'Rangpur' lime rootstock, one of the most widely used in Brazilian orchards (Dornelles, 1988), has shown sensitivity to the nematode *Tylenchulus semipenetrans* and citrus decline, which has intensified the search for more resistant alternatives. With the intensification of the search for resistant varieties, the development and conservation of germplasm have become important alternatives for research.

Additionally, germplasm collection, conservation, and molecular studies are essential to support future research (Maya *et al.*, 2012). Based on updated characterization methods, phenotypic variability can be analyzed more accurately using collected germplasm materials (Rao & Hodgkin, 2002). Multivariate analysis techniques are used to assess diversity among accessions and identify key descriptors for distinguishing accessions in germplasm banks. Among these statistical techniques, principal component analysis and clustering methods stand out (Cruz *et al.*, 2012). Several studies have demonstrated the remarkable antioxidant activity of Tahiti lime juice (Perez *et al.*, 2010; Barreca *et al.*, 2011). However, available information on sweet lemon seed characterization and diversity remains limited. In this context, the evaluation of seed biometric traits may provide important information about the variability among accessions and support the identification of materials with potential use in citrus breeding programs.

Despite the economic and agronomic importance of sweet lemon, information regarding seed biometric variation among accessions remains scarce. Most available studies on citrus have focused on fruit quality, propagation, or molecular characterization, while phenotypic variation in seed traits has received limited attention. This knowledge gap restricts the identification of potentially distinct accessions for germplasm conservation, rootstock selection, and future breeding strategies. Therefore, a systematic evaluation of seed biometric traits is needed to determine the extent of phenotypic diversity present within sweet lemon germplasm collections. This study aimed to characterize seed biometric traits and quantify the phenotypic diversity among nine sweet lemon (*Citrus limetta* Risso) accessions using multivariate statistical analyses. It was hypothesized that significant phenotypic variation exists among accessions and that seed biometric descriptors can effectively discriminate groups with potential value for germplasm conservation and citrus breeding programs.

MATERIALS AND METHODS

Study location

The experiment was conducted at the Agronomic Research Center of the Federal University of Jataí (UFJ), located in the municipality of Jataí, state of Goiás, Brazil (17° 52' 51" S, 51° 42' 50" W).

Plant material collection site

The fruits used in the study were collected from a residential property located in the rural area of the municipality of Porto Nacional, state of Tocantins, Brazil (10° 42’ 28” S, 48° 25’ 01” W). According to the Köppen climate classification, the region has a tropical dry savanna climate (Aw), with average annual temperatures ranging between 28 °C and 29 °C (Alvares *et al.*, 2013).

Nine sweet lemon (*Citrus limetta* Risso) accessions, identified as M1, M2, M3, M4, M5, M6, M7, M8, and M9, were selected. Each accession corresponded to an individual adult tree maintained in the collection area. The selected trees exhibited a uniform growth habit, high productivity, good phytosanitary condition, and adequate nutritional status. All trees originated from sexual propagation, were cultivated under micro-sprinkler irrigation, spaced at 4 × 4 m, and were approximately nine years old.

Fruit collection was carried out during the harvest season, when fruits had reached commercial maturity, as determined by the characteristic yellow peel color and full fruit development. For each accession, 35 fruits were manually harvested at random from different portions of the canopy to ensure representative sampling. Thirty-five fruits were initially collected per accession. After quality screening, 25 fruits were retained for biometric analyses.

Plant material storage

After collection, the fruits were placed in labeled plastic boxes containing a single fruit layer and transported in a non-refrigerated vehicle to the laboratory of the Federal University of Jataí (UFJ), Goiás, Brazil.

In the laboratory, the fruits were washed with running water and dried at room temperature. Subsequently, a visual inspection was conducted, and fruits showing mechanical damage, deformation, symptoms of pests or diseases, or other defects were discarded. After this screening procedure, 25 fruits per accession were retained for biometric evaluations.

The fruits were manually opened, and the seeds were extracted and washed to remove pulp residues. All seeds obtained from the selected fruits were used for biometric characterization. Seed length, width, and thickness were measured using a digital caliper with 0.01 mm resolution, while seed fresh mass was determined using an analytical balance with 0.001 g precision. Equipment calibration followed the manufacturer’s recommendations before data collection.

Experimental design and statistical analysis

The study was conducted in a completely randomized design involving nine accessions (M1 to M9). For biometric analyses, 25 fruits per accession were evaluated. Fruits were considered subsamples within each accession because they originated from the same source tree. Therefore, inference from the results is restricted to the evaluated accessions and should not be interpreted as estimates of variability among sweet lemon populations. Because each accession was represented by a single tree and fruits were treated as subsamples, the ANOVA results should be interpreted as descriptive comparisons among the evaluated accessions rather than as inferential estimates applicable to broader sweet lemon populations.

For the univariate analyses, accession means were subjected to analysis of variance (ANOVA) according to the following model (equation 1):

$$Y_{ij} = \mu + A_i + E_{ij} \qquad \text{e1}$$

where Yij is the observed value, μ is the overall mean, Ai is the effect of the ith accession, and Eij is the experimental error.

Prior to ANOVA, residuals were tested for normality and homogeneity of variances using the Shapiro-Wilk and Bartlett tests, respectively. When significant effects were detected by the F test, means were compared using Tukey's test at the 5% probability level ($p \leq 0.05$).

Descriptive statistics were calculated, including means, minimum and maximum values, and coefficients of variation. Pearson correlation coefficients were estimated among biometric traits to evaluate the magnitude and direction of the associations between variables. The significance of the correlations was tested at the 5% probability level.

To assess phenotypic diversity among accessions, the relative contribution of each trait to total divergence was estimated according to the methodology proposed by Singh (1981). Multivariate analyses were performed using standardized data (mean = 0 and variance = 1) to eliminate scale effects among variables.

Genetic dissimilarity matrices were obtained using the standardized Euclidean distance, and accession grouping was performed through the Tocher optimization method (Cruz *et al*., 2012). Principal component analysis was also used to visualize the phenotypic relationships among accessions. All statistical analyses were performed using Rbio software (version 119, released on June 6, 2019) and GENES software version 5.0 (Cruz, 2013).

RESULTS

Biometric analyses were performed on 225 fruits (25 fruits from each of nine accessions). Because the fruits represented subsamples within individual accession trees, the results should be interpreted as descriptive patterns of phenotypic variation among the evaluated accessions.

The selected fruits weighed 325.6 ± 82.6 g, were 84.5 ± 8.9 mm long, had a diameter of 87.2 ± 8.4 mm, and had a peel hue angle of 92.6 ± 10.4°. The number of seeds per fruit was 16.5 ± 5.5, seed width was 6.5 ± 0.7 mm, and seed length was 11.3 ± 0.9 mm, as shown in Table 1. The values presented represent descriptive means of the evaluated accessions. Because each accession corresponded to a single source tree and fruits were treated as subsamples, statistical comparisons should be interpreted as descriptive patterns of phenotypic variation rather than inferential tests applicable to broader populations.

Table 1. *Mean number of seeds per fruit (NS), seed width (SW), seed thickness (ST), and seed length (SL) of seeds collected from fruits of nine accessions of sweet lemon (C. limetta)*

Accession	NS	SW (mm)	ST (mm)	SL (mm)
1	18.00	6.63	4.78	10.82
2	15.80	6.36	5.20	10.96
3	14.80	6.52	5.17	11.03
4	19.40	5.52	4.63	11.30
5	17.80	6.35	5.46	10.79
6	17.00	7.22	4.96	12.18
7	17.00	6.99	5.38	11.13
8	17.20	6.16	5.52	11.80
9	14.60	6.60	5.02	11.01
CV	35.01	10.37	7.40	8.60

After the fruits were sectioned, the seeds were extracted and evaluated for seed number by direct counting and for seed length, thickness, and width using a digital caliper. The measurements were expressed in mm.

Accession 4 showed the lowest seed width, whereas accessions 6 and 7 presented the highest values (Table 1). Seed thickness ranged from 4.63 to 5.52 mm, and seed length ranged from 10.82 to 12.18 mm (Table 1).

The number of seeds showed high variability among the nine accessions, with an average of 16.51 seeds per fruit, a minimum of 6, a maximum of 29, and a coefficient of variation of 35.01% (Table 2).

Table 2. *Average, minimum and maximum values, coefficient of variation (CV), and relative contribution (s.j) of the features of seeds collected from fruits of nine sweet lemon accessions (C. limetta) to dissimilarity*

Feature	Average	Minimum	Maximum	CV (%)	s.j (%)
NS	16.51	6.00	29	35.01	83.02
SW (mm)	6.48	4.96	8.05	10.37	7.36
SL (mm)	11.22	9.95	13.80	17.60	2.85
ST (mm)	5.12	2.48	7.33	7.40	6.77

Note. NS - Number of seeds per fruit, SW - Seed width, SL - Seed length, and ST - Seed thickness.

Seed length, width, and thickness also varied among the accessions. Seed thickness had the smallest coefficient of variation, 7.4%, indicating the lowest dispersion among the evaluated seed traits (Table 2).

The relative contribution of the traits to dissimilarity among accessions (S.j), estimated using the criteria proposed by Singh (1981), ranged from 2.85% to 83.02%. The variables that contributed most to total dissimilarity were the number of seeds (83.02%), seed width (7.36%), and seed thickness (6.77%) (Table 2).

The phenotypic correlation analysis showed a positive and significant correlation between the number of seeds and seed thickness, as well as a negative and significant correlation between the number of seeds and seed length (Table 3).

Table 3. *Phenotypic correlation coefficients of the features from C. limetta seeds obtained from fruits of nine accessions*

Feature	Number of seeds	Width	Length	Thickness
Number of seeds	1	-0.34^{NS}	-0.53*	0.34*
Width	-0.34^{NS}	1	0.21^{NS}	0.22^{NS}
Length	-0.53*	0.21^{NS}	1	0.00^{NS}
Thickness	0.34*	0.22^{NS}	0.00^{NS}	1

Note. *Significant at $p \leq 0.05$., NS = Not significant ($p > 0.05$). Correlations were calculated using accession means (n = 9).

The grouping by Tocher's method resulted in three distinct groups based on the evaluated traits. Accessions 6 and 4 were individually allocated to groups 2 and 3, respectively, whereas group 1 included the largest number of accessions: 3, 9, 2, 5, 7, 1, and 8 (Table 4).

Table 4. *Groups established by Tocher's method based on the four features from seeds of nine sweet lemon accessions (C. limetta)*

Group	Accession
1	3; 9; 2; 5; 7; 1; 8
2	6
3	4

DISCUSSION

The results indicate that seed biometric traits are useful descriptors for distinguishing sweet lemon accessions and identifying materials with potential value in citrus germplasm management. The peel hue angle values close to 90° indicate a more intense yellow fruit color, while the observed seed values show measurable variation among the evaluated accessions in traits relevant to their characterization.

The number of seeds recorded in *C. limetta* was similar to the values reported for "Cravo Santa Cruz" lemon (*Citrus limonia* Osbeck), with 16 seeds per fruit (Santos *et al.*, 2015), and for 'Sunki Tietê' small-fruited mandarin (*Citrus sunki*), with 14.7 seeds per fruit (Schinor *et al.*, 2011). However, the values differed from those reported for the Sicilian lemon genotype "Genova" (*C. limon*), with 34.56 seeds per fruit (Ferreira *et al.*, 2018), and for Orange BRS Russas 02 (*Citrus sinensis* L. Osbeck), with 3.0 seeds per fruit (Sombra *et al.*, 2018). According to Roverssi *et al.* (2022), higher seed formation may be associated with cross-pollination, which favors both fruit set and seed number.

Seed size is relevant because larger seeds may contain greater energy reserves, which can favor embryo development, germination, vigor, and the production of more uniform seedlings (Carvalho & Nakagawa, 2012; Pagliarini *et al.*, 2014). Thus, accessions 6 and 7 exhibited greater seed width; however, additional studies evaluating germination, seedling vigor, and graft compatibility are required before recommending these materials for nursery production. The greater seed length observed in the present study, compared with values reported for "Cleópatra" and 'Sunki Tietê' mandarins by Schinor *et al.* (2011), also reinforces the existence of morphological variation among citrus genotypes.

The high coefficient of variation for the number of seeds should not be interpreted as experimental imprecision but rather as evidence that the evaluated traits may be influenced by phenotypic and environmental sources of variation (Silva *et al.*, 2012). This variability is agronomically meaningful because seed availability affects the feasibility of using a genotype as a propagation source. In commercial fruit markets, a reduced seed number may be preferred for fresh consumption and processing, whereas in rootstock production, a larger number of viable seeds is advantageous because seedling demand is high (Alcântara *et al.*, 2018; Zucoloto *et al.*, 2011).

The relative contribution analysis showed that the number of seeds was the main variable explaining dissimilarity among the accessions. This result is important because the relative contribution of each trait assists in discarding less informative variables and selecting descriptors for phenotypic divergence studies (Cruz *et al.*, 2012). In this context, the combined evaluation of seed number, seed width, and seed thickness provides a practical set of descriptors for the preliminary screening of sweet lemon germplasm.

The significant phenotypic correlations observed among seed traits also have implications for selection. Phenotypic correlation measures the degree of association between traits and indicates whether changes in one trait may be accompanied by

changes in another (Silva & Schmildt *et al.*, 2016). Therefore, knowledge of these associations may support indirect selection and help breeders define selection criteria for superior genotypes (Correa *et al.*, 2015). The identification and characterization of phenotypic diversity represent initial steps for the use of plant materials in breeding programs and for the conservation of diversity (Dantas *et al.*, 2012).

The Tocher grouping indicated divergence among accessions, particularly because accessions 6 and 4 were placed in isolated groups. This method is useful because it tends to allocate more similar individuals in the first groups and more divergent individuals in the later groups, allowing the identification of phenotypically dissimilar accessions (Silva & Coelho *et al.*, 2016). Groups containing a single accession indicate greater divergence in comparison with the remaining accessions (Barros *et al.*, 2016), whereas the concentration of most accessions in a single group suggests similarity among several materials and may indicate a relatively homogeneous phenotypic profile among the evaluated accessions (Azevedo *et al.*, 2014).

The divergence detected for accessions 4 and 6 reinforces the possibility of selecting contrasting parental materials to broaden the phenotypic diversity base of future phenotypic variability. Divergent individuals may generate greater variability in segregating phenotypic variability, increasing the probability of selecting favorable allele combinations (Nardino *et al.*, 2017). Therefore, the combined interpretation of biometric variables and multivariate grouping provides a practical criterion for sweet lemon conservation, characterization, and selection, especially where molecular data are not yet available or where low-cost descriptors are required for early germplasm screening.

In sweet lemon accessions, seed traits may be especially useful as preliminary descriptors because they are simple to measure, require low-cost equipment, and can be evaluated in large numbers of fruits or accessions. Although biometric descriptors do not replace molecular markers or field performance trials, they provide an accessible first step for identifying materials that deserve more detailed characterization. This is particularly relevant in germplasm collections where many accessions remain insufficiently described and where rapid screening can help prioritize materials for conservation, propagation, or breeding activities.

CONCLUSION

The biometric characterization of *Citrus limetta* seeds revealed significant variability in the number of seeds per fruit, with an average of 16.51 seeds and a wide variation ranging from 6 to 29 seeds. Seed width showed significant differences among accessions, with accession 4 presenting the lowest values. No significant differences were detected in seed thickness or length. Accessions 4 and 6 were the most phenotypically divergent based on four seed traits. Cluster analysis resulted in three distinct groups, highlighting divergent accessions that may constitute promising materials for further evaluation in citrus breeding programs. These results reinforce the importance of seed biometrics in the conservation and exploitation of phenotypic diversity. From an applied perspective, accessions with contrasting seed traits should be prioritized in future evaluations that include seed germination, seedling vigor, nucellar embryony, field performance, and rootstock compatibility. Such complementary analyses may determine whether the biometric divergence observed in this study is associated with agronomic performance, thereby increasing the usefulness of these accessions for citrus breeding and nursery production.

AUTHOR CONTRIBUTIONS

Conceptualization, H.F.A., A.H.S., L.C.C., and D.F.P.S.; Methodology, M.E.S.M., P.M.S., B.S.S., I.S.G., R.F.C., L.H.R.G., A.N.S.N., and R.F.M.; Software, R.F.C., L.H.R.G., and A.N.S.N.; Validation, H.F.A., A.H.S., L.C.C., and D.F.P.S.; Formal Analysis, M.E.S.M., P.M.S., R.F.C., L.H.R.G., and A.N.S.N.; Investigation, M.E.S.M., P.M.S., B.S.S., I.S.G., R.F.C., L.H.R.G., A.N.S.N., and R.F.M.; Resources, H.F.A., L.C.C., and D.F.P.S.; Data Curation, R.F.C. and L.H.R.G.; Writing—Original Draft Preparation, M.E.S.M., P.M.S., B.S.S., I.S.G., R.F.C., L.H.R.G., A.N.S.N., and R.F.M.; Writing—Review & Editing, H.F.A., A.H.S., L.C.C., and D.F.P.S.; Visualization, M.E.S.M., P.M.S., R.F.C., and L.H.R.G.; Supervision, H.F.A., A.H.S., L.C.C., and D.F.P.S.; Project Administration, L.C.C. and D.F.P.S.; Funding Acquisition, L.C.C. and D.F.P.S. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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