

Original Article

Analysis of molecular variability of “Tahiti” acid lime selections

Análise da variabilidade molecular de seleções de limeira ácida “Tahiti”

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Abstract

The ‘Tahiti’ acid lime (*Citrus latifolia* Tan.) is one of the main fruit trees produced in Brazil, but its triploid nature poses challenges for the genetic improvement of the crop. The aim of this study was to molecularly characterize selections of ‘Tahiti’ acid lime plants by quantifying DNA methylation and genetic differentiation using microsatellite markers, in order to support work on the conservation and genetic improvement of the crop. We used 12 plants considered to be mutants, previously selected for their distinct biometric characteristics. After extracting the genomic DNA, molecular analyses were carried out to measure the relative quantification of global methylation between the individuals. The experimental design used was completely randomized, with 12 treatments and 4 replications. Post-hoc analysis of the data was carried out using the Tukey test at 5%. Molecular analysis was then carried out using 12 pairs of microsatellite molecular markers. Differences were observed in the DNA methylation patterns between the treatments evaluated, especially treatments 3 and 9, which showed absorbance values of 0.621 and 0.266 nm, respectively. With regard to the analysis with microsatellite markers, it was possible to amplify 19 bands, but all were monomorphic, indicating that the different phenotypes observed may be due to alterations in the epigenome of these plants.

Keywords: *Citrus latifolia* Tan., citrus farming, spontaneous mutation, DNA methylation, epigenetics.

Resumo

A lima ácida ‘Tahiti’ (*Citrus latifolia* Tan.) está entre as principais frutíferas produzidas pelo Brasil, porém, sua natureza triploide traz desafios para melhoramento genético da cultura. O presente trabalho tem como objetivo a caracterização molecular de seleções de plantas lima ácida ‘Tahiti’, por meio da quantificação da metilação do DNA e diferenciação genética por marcadores microsatélites, a fim de subsidiar trabalhos de conservação e melhoramento genético da cultura. Foram utilizadas 12 plantas tidas como mutantes, previamente selecionadas por suas características biométricas distintas. Após a extração do DNA genômico, foram realizadas análises moleculares para mensurar a quantificação relativa de metilação global entre os indivíduos. O delineamento experimental utilizado foi o inteiramente casualizado, com 12 tratamentos e 4 repetições. A análise *post-hoc* dos dados foi realizada pelo teste de Tukey a 5%. Na sequência, realizou-se análises moleculares com 12 pares de marcadores moleculares microsatélites. Foram observadas diferenças nos padrões de metilação dos DNAs entre os tratamentos avaliados, com destaque para os tratamentos 3 e 9, que apresentaram valores de absorbância de 0,621 e 0,266 nm, respectivamente. Com relação às análises com marcadores microsatélites, foi possível à amplificação de 19 bandas, porém todas monomórficas, indicando que os diferentes fenótipos observados podem ser devido a alterações no epigenoma dessas plantas.

Palavras-chave: *Citrus latifolia* Tan., cultivo de citros, mutação espontânea, metilação de DNA, epigenética.

1. Introduction

Brazilian citrus farming, being an essential pillar of the country’s agricultural economy, establishes Brazil as the global leader in the production of citrus fruits, with emphasis on the production of ‘Tahiti’ acid lime (*Citrus latifolia* Tan.) (Costa et al., 2020). This fruit

not only drives socioeconomic development in rural areas, but also significantly contributes to the food and nutritional security of the population, being a rich source of vitamin C, antioxidants and other crucial nutrients (Saini et al., 2015).

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The global market for citrus fruits, including ‘Tahiti’ acid lime, continues to grow. This growth is driven by the increasing demand for healthy foods and the fruit versatility in various food and beverage industries (Saini et al., 2015). In 2023, the production of ‘Tahiti’ acid lime remained high in Brazil, with advances in irrigation and agricultural management techniques that have improved production efficiency, significantly contributing to the sustainability and competitiveness of national production (Mahmoud et al., 2023).

However, Brazilian citrus farming faces notable challenges, especially with regard to plant genetic improvement. In the case of the ‘Tahiti’ acid lime, as it is a triploid species, the application of traditional breeding techniques such as hybridization is difficult due to its complex genetic structure (Soares Filho et al., 2013). In addition, this crop is vulnerable to several diseases and pests, such as citrus canker caused by *Xanthomonas citri* subsp. *citri*, and Greening (*Huanglongbing*), which can result in considerable production losses (Bassanezi et al., 2020). Thus, the exploration of spontaneous mutations becomes a fundamental strategy for the creation of new cultivars with characteristics of agronomic interest, contributing to the genetic variability necessary in breeding programs (Kondratieva et al., 2020).

In this sense, plants, as sessile organisms, are often exposed to unavoidable environmental conditions. Stress responses to these conditions are multivariate (Claeys and Inzé, 2013), which characterizes the meaning of the expression phenotypic plasticity, requiring its biometric and molecular characterization, which, when integrated, provides a more complete and precise approach for the selection of mutants with great potential for the development of new cultivars (Novelli et al., 2006).

DNA methylation, an epigenetic mechanism, can affect gene expression and, consequently, the phenotypic characteristics of plants (Wu et al., 2022; Rodrigues et al., 2022). Studies have shown the link between DNA methylation and characteristics of agronomic interest in citrus, such as disease resistance (Liang et al., 2020) and fruit quality (Huang et al., 2023). Furthermore, analysis with molecular markers, such as microsatellites (SSRs), has proven to be a very powerful tool for identifying and characterizing mutants in citrus (Luro et al., 2008).

In this sense, the present work aims to characterize molecular selections of ‘Tahiti’ acid lime plants by quantifying DNA methylation and genetic differentiation using microsatellite markers in order to support conservation and genetic improvement works with the crop.

2. Material and Methods

The experiment was developed using previously selected ‘Tahiti’ acid lime plants from the Costa Mello Farm, located in the municipality of Iturama - MG, whose geographic coordinates are 19°43’40” S and 50°11’45” W, with average altitude of 453 meters a.s.l. The predominant climate is semi-humid tropical, with average annual rainfall of 1378 mm, concentrated between November and March and average annual temperature of 24.7 °C (Santos and Vazquez, 2020).

Previously, in a commercial ‘Tahiti’ acid lime orchard, 12 canopies of ‘Tahiti’ acid lime plants morphologically distinct from the other IAC-5 clones were observed in the area, with fruits exhibiting light green to yellowish coloration, some showing a completely yellow peel even at the commercial stage; variations in fruit shape, including round, oval, and slightly oblong forms; a smoother peel surface in some fruits; and leaves with narrower blades, lanceolate shape, irregular margins, and a more elongated and acute apex, being characterized as probable spontaneous mutants. These 12 canopies were grafted onto Fly Dragon rootstock (*Poncirus trifoliata* var. *monstrosa*), and grown with spacing of 3 meters between rows and 2 meters between plants, with average production age of 10 years.

Regarding cultural practices, irrigation is being managed by central pivots, with intermittent operation guided by management strategies and climate variables. Pest and disease control was conducted through the careful application of registered products, according to seasonal and preventive needs. Fertilization and soil correction are carried out after specific soil and leaf analyses for each area. No pruning was carried out in the production areas.

To extract genomic DNA from plants, using the CTAB method, young leaves from each selected plant were selected, packaged in thermal boxes and taken to the Fruit and Floriculture Laboratory of the Faculty of Agricultural and Technological Sciences (FCAT/UNESP), Dracena Campus - SP, where molecular analyses were carried out.

After extraction, quantification was performed with 1 µL of the extracted genomic DNA in spectrophotometer (NanoDrop 2000, Thermo Scientific), based on the 260 - 280 nm ratios (> 1.8) for contamination by proteins and 260 - 230 nm (> 2.0) for contamination by phenols and polysaccharides, using ultra-pure water for the initial adjustment of the equipment following the manufacturer’s recommendations (Sambrook et al., 1989), whose quantification values can be observed in Table 1.

Table 1. DNA quantification of spontaneous ‘Tahiti’ acid lime mutants by spectrophotometry.

Treatments	260 nm / 280 nm ratio	Concentration (ng/µL)
1	1.87	274.2
2	1.95	109.8
3	1.97	136.2
4	1.93	124.4
5	1.95	297.0
6	1.88	327.7
7	1.95	256.8
8	1.93	283.9
9	1.88	219.5
10	1.88	242.0
11	1.88	331.4
12	1.88	334.5

The ELISA (Enzyme-Linked Immunosorbent Assay) test, a methodology essentially based on the use of antibodies sensitive to 5-mC, was used to perform the relative quantification of global methylation between the genomic DNAs of treatments. This procedure was performed using the Imprint DNA Methylation Quantification kit (Sigma-Aldrick), using strips with wells pre- treated with methylated DNA binding reagent, and using a capture antibody sensitive to DNA methylation and a detection antibody, allowing the colorimetric detection of the absolute amount of methylation present in the DNA.

The experimental design used was completely randomized with 12 treatments, with each selected plant considered as a treatment, in triplicate. The absorbance of the solution contained in wells was performed at wavelength of 450 nm in a PowerWav XS Microplate Reader (Biotek).

The differences in the quantification of DNA methylation content between treatments were evaluated using Analysis of Variance (ANOVA). The post-hoc analysis was performed with the Tukey test at 95% confidence level ($p < 0.05$) using the SISVAR software version 5.6 (Ferreira, 2019).

Subsequently, for the *loci* analysis, the amplification of microsatellite regions (SSR) was performed using 12 pairs of primers (Table 2).

The 15 μ L volume amplification reaction consisted of PCR buffer (1X), 0.1 mM of each dNTP, 2 mM $MgCl_2$, 1 μ M of each member of the primer pair (F and R), 2 units of Taq DNA polymerase, 18 ng of genomic DNA. The amplification conditions were as follows: 3 min at 94 °C (denaturation), 30 sec at 94 °C, 1 min at the annealing temperature of each primer pair (Table 2), 1 min at 72 °C, 35x (30 sec at 94 °C, 1 min at the annealing temperature of each primer pair, 1 min at 72 °C) and 10 min at 72 °C (extension), performed in the Veriti 96-Well Thermal Cyclers (Applied Biosystems).

Amplification products were separated on a 12% polyacrylamide denaturing gel prepared with 120 mL of polyacrylamide matrix, 120 μ L of TEMED, and 800 μ L of APS (95 mg/mL) in 1X TBE buffer. Electrophoresis was performed at constant current of 80 W and maximum temperature of 50 °C for four hours.

Gels were stained by photorevelation of polyacrylamide plates using solution containing glacial acetic acid for the fixation step; solution containing nitric acid for the pretreatment step; silver nitrate solution for the impregnation step; and solution containing anhydrous sodium carbonate and formaldehyde for gel development, following the protocol described by Creste et al. (2001). The results of samples after electrophoresis were visualized by visual reading, followed by counting of bands to obtain a binary matrix.

3. Results and Discussion

The quantification of global DNA methylation, in Table 3, revealed statistically significant variation among the spontaneous ‘Tahiti’ acid lime selections. Notably, treatment 3 presented the highest absorbance value (0.621), indicating a substantially elevated level of 5- methylcytosine and suggesting broad transcriptional repression potentially linked to the altered phenotype observed. Treatment 9 also exhibited a relatively high absorbance (0.266), differing

statistically from most other selections, which may reflect moderate epigenetic modulation. These findings imply that spontaneous mutations in ‘Tahiti’ acid lime may be accompanied by distinct epigenetic reprogramming events, contributing to phenotypic variability through the regulation of gene expression at the DNA methylation level.

Table 4 presents the number of fragments amplified by the 12 pairs of microsatellite molecular marker primes used, observing a total of 19 monomorphic bands generated.

The absence of polymorphism in SSR markers in spontaneous mutants was observed in several citrus species, including ‘Pêra’ orange (Martasari et al., 2023), which can be explained by the clonal nature of the propagation of these plants, which maintains genetic uniformity among individuals (Barkley et al., 2006).

Rodrigues et al. (2012) investigated the genetic variability in fig mutants originated by gamma radiation, with RAPD and AFLP markers, and found that of the more than a thousand amplified fragments, none showed polymorphism, supporting the hypothesis that the absence of polymorphism in molecular markers in mutants may be a common characteristic in clonal plants, due to the genetic stability maintained during vegetative propagation.

Furthermore, the lack of polymorphism in SSRs can also be attributed to the low spontaneous mutation rate in microsatellite regions of the genome (Vigouroux et al., 2002). However, the possibility of divergences between these materials cannot be ruled out, since there is evidence of somatic mutation in citrus occurring at high rate, which gave rise to closely related individuals (Folegatti et al., 2005), but that molecular markers cannot always detect (Barkley et al., 2006).

In this case, the absence of polymorphism in SSR markers, combined with variation in the quantification of global DNA methylation content, suggests that the phenotypic variability observed in spontaneous ‘Tahiti’ acid lime mutants may be the result of epigenetic mechanisms, since DNA methylation is an epigenetic mechanism that can influence gene expression without altering the genomic sequence, resulting in different phenotypes (Wu et al., 2022; Rodrigues et al., 2022).

Table 2. SSR primers used in citrus amplification, with their respective sequences and annealing temperatures (Ta).

Nome	Sequence (5'-3')	Ta °C
CTR 1	CACACACACACACAR'G	55
CTR 2	AACAACAACAACARC ¹	48
CTR 3	ATCATCATCATCATCRC ¹	48
CTR 4	ATGATGATGATGATGRC ¹	48
CTR 5	CAACAACAACAACARC ¹	48
CTR 6	CTTCTTCTTCTTCTTRC ¹	48
CTR 7	TGTTGTTGTTGTTGTYC ²	48
CTR 8	CRTGTTGTTGTTGTTGT ¹	48
CTR 9	AGAAGAAGAAGAAGARC ¹	48
CTR 10	TGTTGTTGTTGTTGRC ¹	47
CTR 11	CCGCCCGCCGCCGRC ¹	72
CTR 12	GTAGTAGTAGTAGTARC ¹	48

¹R= (A,G); ²Y= (C,T).

Table 3. DNA absorbance values of spontaneous ‘Tahiti’ acid lime mutants obtained by ELISA test with anti-5-mC antibodies and their respective analysis of variance.

Source of variation	Absorbance 450nm			Mean square
Treatments	Replicate 1	Replicate 2	Replicate 3	0.071688*
1	0.086	0.078	0.085	0.083 f
2	0.075	0.072	0.081	0.076 f
3	0.641	0.606	0.617	0.621 a
4	0.190	0.186	0.195	0.190 c
5	0.163	0.160	0.163	0.162 d
6	0.163	0.172	0.164	0.166 d
7	0.137	0.149	0.144	0.143 d
8	0.075	0.064	0.072	0.070 f
9	0.263	0.252	0.283	0.266 b
10	0.076	0.070	0.082	0.076 f
11	0.064	0.062	0.075	0.067 f
12	0.111	0.110	0.126	0.115 e
Mean				0.167
CV %				4.59

Coefficient of Variation (CV). *Significant by the Tukey’s test at 5% probability. Different letters statistically differ from each other.

This assumption is supported by studies in other plant species that show the importance of epigenetics in generating phenotypic diversity, even in the absence of genetic variation (Schmitz et al., 2011; Rodrigues et al., 2015). In the case of ‘Tahiti’ acid lime, DNA methylation may be impacting the expression of important genes, such as those related to fruit size and shape, peel color and the production of volatile compounds, resulting in the different phenotypes observed.

Studies in other citrus species have shown the connection between DNA methylation patterns and important agronomic traits, such as disease resistance (Liang et al., 2020), fruit ripening (Huang et al., 2023) and fruit quality, including soluble solids content and acidity (Zhang et al., 2022). Furthermore, DNA methylation has been observed to be linked to the regulation of crucial processes in plant development, such as flowering and fruiting (Zhang et al., 2018).

The results of this study open new perspectives for the genetic improvement of the ‘Tahiti’ acid lime tree, highlighting the potential of DNA methylation analysis as a tool complementary to the analysis of molecular markers. The identification of epigenetic markers associated with desirable traits can aid in the selection of mutants with potential to develop new cultivars, thus contributing to the sustainability and competitiveness of the Brazilian citrus farming.

In addition, understanding the epigenetic mechanisms involved in the regulation of gene expression in citrus can lead to the development of new breeding strategies, by manipulating DNA methylation to induce desirable traits in cultivated plants (Wu et al., 2022). Epigenetics, together with traditional genetics, can offer new tools that could be used in the development of more productive and resistant ‘Tahiti’ acid lime cultivars adapted to market demands and climate change.

Table 4. Number of bands generated by the amplification of DNA fragments from selected ‘Tahiti’ acid lime plants by 12 pairs of SSR molecular marker primers.

Pair of SSR Primers	NM	NP
CTR 1	1	0
CTR 2	1	0
CTR 3	1	0
CTR 4	1	0
CTR 5	2	0
CTR 6	1	0
CTR 7	2	0
CTR 8	3	0
CTR 9	2	0
CTR 10	2	0
CTR 11	1	0
CTR 12	2	0
TOTAL	19	0

NM = number of monomorphic bands; NP = number of polymorphic bands.

4. Conclusion

Was a significant difference between quantification values of the global DNA methylation content among selected ‘Tahiti’ acid lime plants, suggesting a phenotypic variation by gene expression, since no variations were observed in the DNA sequence of these plants using SSR molecular markers.

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Data Availability Statement

All data used and analyzed in this study are fully presented in this published article. Additional information, including raw data and detailed processing procedures, is not publicly available for technical and ethical reasons but may be provided by the corresponding author upon reasonable request.

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