Epigenetic variation in guava (*Psidium guajava*) genotypes during the vegetative and reproductive phases of the production cycle

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ABSTRACT. *Psidium guajava* (guava) is a fruit tree of the Myrtaceae family of nutritional and economic importance and wide edaphoclimatic adaptation. For commercial orchard purposes, guava trees are derived from clonal propagation and the production cycle begins with drastic pruning. During its development the guava tree displays varied characteristics that may involve epigenetic mechanisms, such as DNA methylation, revealed by the formation of 5-methylcytosine (5-mC). In order to understand the variability of this mechanism, the %5-mC was evaluated in 22 guava genotypes from a working collection, in an experimental orchard, over five development stages – post-pruning, flowering, fructification, harvest and post-harvest – and subsequently in different tissues. Wide variation in DNA methylation was detected among the genotypes. Hypermethylation was found in one, whereas methylation and demethylation mechanisms were observed in 10 genotypes. Greater variability in DNA methylation among genotypes was found during the vegetative phase, mainly after pruning. On the other hand, uniformity was observed among the genotypes with regards to global DNA methylation during flowering, indicating that this mechanism is conserved in this specific phase. No differences in global DNA methylation was detected in the different plant tissues. In this study, the global epigenetic mechanisms of *P. guajava* were detected more frequently in vegetative than in reproductive phases. Important

Brazilian cultivars such as Paluma, Pedro Sato, Sassaoka and Cortibel SLG exhibit changes in their methylation profiles during their production cycle. Others show greater stability, for instance Século XXI and six Cortibel cultivars, suggesting the possibility of selection for this characteristic. Furthermore, the methylation and demethylation mechanisms of adult guava plants indicate the relevance of epigenetics for the wide edaphoclimatic adaptation of the species. In addition, the clonal propagation of guava may provide an opportunity for the development of this crop through epibreeding approaches.

Key words: Methylation; Guava tree; 5-mC; Gene expression; HPLC

INTRODUCTION

In plants, epigenetic variation contributes to phenotypic differences in developmental traits (Turck and Coupland, 2013; Alonso et al., 2018). Considering this, epigenetic diversity may provide new targets for breeding and crop improvement programs searching for novel alternatives in view of the reduced genetic variability brought about by intensive agriculture (Gallusci et al., 2017; Latutrie et al., 2019). The influence of epigenetic mechanisms has been reported for plant development, and responses to biotic and abiotic stresses and salinity in annual plants (Tiwari et al., 2015; Georgieva et al., 2017; Wang et al., 2018), as well as intraspecific variations in natural populations (Alonso et al., 2018). However, knowledge about epigenetic variation in genotypes for selection is still incipient. Considering this, we focused on the perennial crop species guava (*Psidium guajava*), using commercial orchards established from young trees obtained via clonal propagation.

The guava tree is the most economically important crop in the genus *Psidium* (Myrtaceae). The guava fruit has high vitamin C content, expressive percentages of sugar, vitamin A and B, iron, phosphorus and calcium, and is both consumed *in natura* and used in the industry for the production of juices, sweets and derivatives. The species is native to Mexico and Central America, being cultivated in all tropical and subtropical regions of the world (Silva et al., 2014). In Brazil, an important guava producer, several studies have been conducted using a working collection containing 22 commercial and improved germplasm of guava trees genotypes. These studies revealed phenotypic variability in the vegetative and reproductive development of these plants when grown under the same field conditions (Silva, 2017a), as well as general and specific genotype adaptability (Silva, 2017b), and genetic diversity by molecular markers and agronomic characters (Coser et al., 2012). However, epigenetic mechanisms associated with variability in these genotypes are still unknown.

During plant development, phenotypic variance is attributed to gene expression. Yet, phenotypic plasticity can also be related to epigenetic factors, which may contribute to the adaptation of organisms to different environmental conditions (Gallusci et al., 2017; Lele et al., 2018). Considering this, epigenetic variations represent a new source of phenotypic variance to be used in genetic improvement. In addition, they can be used as epi-marks for the generation and selection of plants, for instance through increase in methylation under grafting conditions as reported for *Cucurbitaceae* (Avramidou et al., 2015) and *Solanaceae* species (Wu et al., 2013). Nevertheless, in order to be explored, such epigenetic variations must be inheritable or stable, which is favored in plants obtained via

clonal propagation, such as the guava tree, since the transmission of epigenetic marks seems to be more stable through mitosis than meiosis (Gallusci et al., 2017; Latutrie et al., 2019).

Epigenetic regulation is referred to as changes in gene function that do not involve modifications in the DNA sequence, being usually accomplished by DNA methylation, histone modifications and chromatin remodeling (Xu et al., 2018). DNA methylation is an important epigenetic mechanism that is involved in transposable element (TE) silencing, genome stability and genomic imprinting. This mechanism consists in the addition of a methyl group at the C5 position of cytosine in order to form 5-methylcytosine (5-mC) (Richards et al., 2017). DNA cytosine methylation is a widespread epigenetic mechanism in eukaryotes, and plant genomes are generally densely methylated. Genomic methylation can be associated with functional consequences such as mutational events, genomic instability, or altered gene expression (Alonso et al., 2015). DNA methylation in promoters has been shown to regulate gene expression and plays a critical role in the growth and development of plants and mammals. In general, DNA methylation is rather diverse among species, occurring under CG, CHG and CHH (where H = A, C, or T) contexts of genes and TEs in angiosperms (Bartels et al., 2018).

Global analyses of 5-mC in the genome have been carried out in phylogenetic studies (Alonso et al., 2015, 2018); in association with the *in vitro* development of plants and fruits (Msogoya et al. 2011); with regard to the influence on phenotypic traits (Novero et al., 2012); in the activation of transposable elements in plant regeneration (Orlowska et al., 2016); and in the effects on chemical metals (Rozhon et al., 2008). Although the global analysis does not distinguish between coding and non-coding sequences and cannot detect subtle differences between individual genes, it enables large-scale studies and evaluation of plant responses, for instance to stress, conditioning and acclimatization conditions (Johnston et al., 2005).

Among the methodologies used to detect DNA methylation, the global 5-mC analysis using High Performance Liquid Chromatography (HPLC) stands out for its reproducibility and precision, being considered the gold standard for quantification and estimation of global DNA methylation (Lisanti et al., 2013; Alonso et al., 2016). It can be used to detect total DNA methylation levels and characterize changes during growth and development (Chen et al., 2013).

Considering the above, we sought to determine whether epigenetic mechanisms act in a significant manner during the phases of vegetative and reproductive development in adult guava trees in the field. Further, we looked for differences in behavior among genotypes regarding the global profile of cytosine methylation, and the methylation profile in different plant tissues. We also compared the genotypes at each development phase and the differences among the developmental phases for each genotype.

MATERIAL AND METHODS

Experimental design and plant material

The experimental orchard was established in the community of Palmeira, located in the city of Mimoso do Sul - Espírito Santo (latitude 21°01'12.99" S, longitude 41°17'13.48" W, altitude 250 m). The orchard was created in March 2012 using a randomized block design with 22 guava genotypes (Table 1) and spacing of 6.0 x 4.0 m. The plants were arranged in three blocks, each containing two plants, resulting in 44 plants per block and 132 plants in total. For 5-mC analysis, the first plants of each genotype were used per block.

Leaf tissue was collected from plants starting from 38 months after planting, at the second pruning for production. The leaves were sampled at five phases, counted in days after pruning (DAP) – 54 DAP, 91 DAP, 119 DAP, 182 DAP and 302 DAP – representing the

respective plant periods: post-pruning, flowering, fructification, harvest and post-harvest. Fully developed leaves were collected around the plant at the same time, packed in liquid nitrogen for transport and then stored at -80°C.

The variation in global 5-mC considering different vegetative and reproductive organs was also analyzed. Samples of young and developed leaves, branch, apical bud and floral bud were collected in five genotypes aged 68 months (Cortibel Rugosa Média, Cortibel XI, Cortibel Rugosa Grande, Paluma and Século XXI). The samples were collected in the same experimental orchard, following the same design and on the same day.

Table 1. Description of the 22 guava genotypes.

Genotype	Abreviation	Type	Pulp color*	Bark texture *	Fruit mass(g)*	Pulp mass (g)*	Pulp Yield (%)*
Cortibel Lisa Grande	CLG	Commercial	Red	Smooth	206.9	148.1	73.1
Cortibel Lisa Média	CLM	Commercial	Red	Smooth	143	106.2	74
Cortibel III	CIII	Massal Selection	Red	Rough	155	110.4	70.7
Cortibel Branca Lisa Grande	CBLG	Commercial	White	Smooth	164.4	121	72.7
Cortibel V	CV	Massal Selection	Red	Semi- Smooth	129.9	93.4	71.7
Cortibel Rugosa Média	CRM	Commercial	Red	Rough	174.8	132.4	74.9
Cortibel VII	CVII	Massal Selection	Red	Rough	159.9	115.1	71.9
Cortibel Branca Rugosa Média	CBRM	Commercial	White	Rough	167.9	122	72.4
Cortibel IX	CIX	Massal Selection	Red	Rough	173.3	131.5	75.4
Cortibel X	CX	Massal Selection	Red	Semi- Smooth	135.4	97.89	71.8
Cortibel XI	CXI	Massal Selection	Red	Semi- Smooth	172	120.1	70.3
Cortibel XII	CXII	Massal Selection	White	Rough	132.4	85.6	65.5
Cortibel XIII	CXIII	Massal Selection	Red	Semi- Smooth	145.5	106.4	72
Cortibel Rugosa Grande	CRG	Commercial	Red	Rough	193.6	145.3	74.5
Cortibel Semi-Lisa Grande	CSLG	Commercial	Red	smooth	193.9	148.1	76.7
Cortibel XVI	CXVI	Massal Selection	White	smooth	149	111.4	74
Cortibel XVII	CXVII	Massal Selection	Red	Semi- Smooth	162.8	121.8	74.3
Sassaoka	SSK	Commercial	Red	Rough	140.3	98.46	70.1
Pedro Sato	PS	Commercial	Red	Semi- Smooth	180.7	134.6	73.9
Século XXI	SXXI	Commercial	Red	Semi- Smooth	165.8	117.5	70.4
Paluma	Plm	Commercial	Red	Semi- Smooth	153.4	107.8	70.3
Roxa	Roxa	Commercial	Purple	Rough	102.6	71.3	69.5

*Variables pulp color, bark texture, fruit mass, mass and pulp yield were described as per Silva (2017b).

Analysis of 5-methylcytosine by HPLC

Extraction of genomic DNA

In order to extract the genomic DNA, the protocol described by Doyle and Doyle (1990) was used, with modifications. The plant tissue was macerated in liquid nitrogen and homogenized in 700 µL of extraction buffer solution (1.4 mol/L NaCl, 100 mmol/L Tris-HCl pH 8.0, 20 mmol/L EDTA pH 8.0, 1% PVP, 2% CTAB, 0.2% β-mercaptoethanol, reverse osmosis water) and incubated at 37°C in dry bath for 30 min. Afterwards, 650 µL of chloroform / isoamyl alcohol (CIA) were added, homogenized by hand for 10 min and centrifuged for 9 min at 10,000 rpm. The supernatant was transferred to a 2.0-mL tube. A total of 200 µL of extraction buffer and 650 µL of CIA were added, and the mixture was homogenized again for 5 min. The

solution was centrifuged for another 9 min at 10,000 rpm. Subsequently, the aqueous phase was transferred to a 1.5-mL tube, 620 μ L of CIA were added and the mixture was centrifuged for further 9 min at 10,000 rpm. The supernatant was transferred to new 1.5-mL tubes and the DNA was precipitated with 500 μ L of ice-cold isopropanol plus 230 μ L of ammonium acetate, being homogenized for 5 min. The mixture was centrifuged at 10,000 rpm for 9 min. At this stage, the pellet was formed and the supernatant was discarded. Using 250 μ L of 70% ethanol, the pellet was washed three times by centrifugation for 3 min at 12,000 rpm for each wash. The pellet was then dried by incubation for 1.5 h at 37°C. To eliminate RNA remnants, the DNA was resuspended in 30 μ L Tris-EDTA with RNase (40 μ g/mL) and kept in water bath at 37°C for 30 min. DNA quality and concentration were estimated using a spectrophotometer NanoDrop® with absorbance at the 260/280 ratio in the range of 1.8 to 2.0.

High Performance Liquid Chromatography (HPLC)

HPLC was used as a global methodology to quantify the 5-mC content in the guava leaves collected at various periods. Hence, after the quantification step, the genomic DNA was adjusted to an amount of 30 μ g of DNA in 100 μ L of water. Acid hydrolysis of the obtained DNA was performed by addition of 50 μ L of 70% (v/v) perchloric acid and heating at 100°C in water bath for 60 min, with subsequent adjustment of the pH to 3-5 (Chen et al., 2013).

Chromatographic analysis was performed using a high performance liquid chromatography system (model Shimadzu LC-20AT), a photodiode array detector (Shimadzu SPD-M20A) and C₁₈ stainless steel column (Shimadzu VP-ODS Shim-pack 250 mm x 4.6 mm I.D., 5- μ m particle size). The mobile phase was isocratic and consisted of aqueous solution of 50 mmol/L KH₂PO₄ (pH 5.8), flow rate of 0.5 mL min⁻¹. The wavelength used was 285 nm (Chen et al., 2013). After the analysis, the chromatographic system was flushed with ultrapure water flow (18.2 M Ω .cm) of 0.5 mL min⁻¹ for 15 min, followed by 90/10 (v/v) methanol / ultrapure water for 15 min at the same flow rate.

Quantification of cytosine (C) and 5-mC was performed by external standard method. Stock standard solutions of C and 5-mC were prepared using standards with 99% purity (Sigma-Aldrich), for final concentrations of 400 μ mol/L for cytosine and 20 μ mol/L for 5-methylcytosine. From these solutions, working solutions were prepared in order to establish the standard curve, using six concentrations ranging from 1 μ mol/L to 30 μ mol/L of C and from 0.25 μ mol/L to 9 μ mol/L of 5-mC. The percentage of 5-mC in the DNA was calculated according to the equation: %5-mC = [5-mC / (C + 5-mC)] x 100.

Statistical analyses

To evaluate methylation, a factorial scheme of 22 x 5 (Factor 1: genotypes; Factor 2: development phase) was used with one plant per repetition. The collected data were analyzed via analysis of variance, and means were compared by Tukey's test for development phase and Scott-Knott for genotypes, both at 5% probability level.

The data were standardized and subjected to multivariate analysis, calculating the dissimilarity matrix for the 22 genotypes, with %5-mC data from the five collection periods, by the Mahalanobis method. The genotypes were subsequently grouped by the Unweighted Pair Group Method (UPGMA) (Sneath and Sokal, 1973). The results are presented in heatmap format. The statistical analyses were performed using the free software environment R (2018) and the ExpDes.pt package.

RESULTS

The analyses revealed that the guava genotypes showed epigenetic variation, with significant differences in relation to the mean %5-mC of the five studied phases. Four groups of genotypes were demonstrated by Scott-Knott test, varying between 16.34% and 26.14% of 5-mC (Figure 1). The cultivar Sassaoka showed hypermethylation compared to the other genotypes. Most commercial cultivars (CLG, CLM, CII, CRM, CXVI, Pedro Sato, Roxa and Século XXI) formed the group with second highest %5-mC. The genotypes CBLG, CV, CBRM, CIX, CX, CXI, CXII CRG, CSLG and Paluma formed the third group, whereas CVII, CXIII and CXVII were the most hypomethylated. The genotypes Sassaoka, Cortibel X, Pedro Sato and Roxa had the highest standard deviations, demonstrating a higher variation of the studied epigenetic mechanism in these genotypes during the production cycle (Figure 1).

Regarding the studied stages of the production cycle (post-pruning, flowering, fructification, harvesting and post-harvesting), the vegetative phase (post-pruning and post-harvest) had the highest standard deviations for mean %5-mC of the 22 genotypes studied (Figure 2). There was significant interaction between the genotypes and the development phases. This indicates that the behavior of genotypes regarding methylation levels varies according to the development phase, and that the effects of development stages on methylation levels are different depending on the genotype studied.

Statistical differences in cytosine methylation levels throughout the plant development were found among genotypes at all stages, except flowering (Figure 2). In the post-pruning phase, a period of intense vegetative growth, the genotypes showed the greatest variation in global 5-mC (from 13.70% to 35.78%), giving rise to four groups of genotypes according to the Scott-Knott test. The first group was formed by the Sassaoka genotype (hypermethylated at this phase with 35.78% of 5-mC). The second group was formed by the genotypes Pedro Sato and Roxa.

Figure 1. Mean %5-mC in the 22 analyzed guava genotypes, considering the five phases studied (post-pruning, flowering, fructification, harvest and post-harvest). The bars indicate the standard deviations obtained with three replicates in five collections during the vegetative and reproductive phases, totaling 15 data per genotype. Means followed by the same letter do not differ by Scott-Knott test at 5% significance level. Genotype abbreviations and descriptions are provided in Table 1.

Figure 2. Analysis of genotypes within each level of development. Boxplot analyses represent the global percentage of 5-methylcytosine for the genotypes analyzed in each phase, with the standard deviations and their respective test of means obtained by Scott-Knott test at a significance level of 5%. Genotype abbreviations and descriptions are provided in Table 1.

During the flowering, fructification and harvest phases, higher homogeneity was observed among the genotypes regarding the studied epigenetic variable. There was no variation among the genotypes during the flowering stage, whereas during fructification and harvest only two groups of genotypes were formed (Figure 3).

During the vegetative post-harvest period, the genotypes showed higher variation compared to the reproductive period, and three groups of genotypes were formed. Most genotypes were in the group with the highest means, ranging from 19.16% to 24.65%. The white cultivars CBLG and CBRM as well as the genotypes CVII and CXVII formed a group. Moreover, the genotypes CX and CXIII presented the lowest methylation condition of the whole experiment at this stage, with 10.94% and 10.66%, respectively. The genotype CVII showed low methylation values during all phases, except fructification, a behavior similar to that of CXVII.

Of the 22 evaluated genotypes, 10 presented significant interaction between phases (Figure 3), which demonstrated two distinct behaviors. In the first, the genotypes presented a lower value of %5-mC during post-pruning, with an increase in reproductive phases (genotypes CVII, CX, CXII, CXIII and CSLG). In the second, the highest %5-mC occurred during post-pruning (Cortibel XVI, Sassaoka, Paluma, Pedro Sato and Roxa). The

genotypes CLG, CLM, CIII, CBLG, CV, CRM, CBRM, CIX, CXI, CRG, CXVII and Século XXI showed no difference in the percentage of 5-mC among development phases.

Figure 3. Genotypes that showed interaction between development phases and 5-mC content. Means followed by the same letter do not differ by Scott-Knott test at 1% significance level. Genotype abbreviations and descriptions are provided in Table 1.

Groups of genotypes with similar behavior as regards DNA methylation in the experiment can be observed in the heatmap organized according to the UPGMA cluster. Two main groups were distinguished: one constituted by the commercial cultivars Sassaoka, Pedro Sato and Roxa, and the other formed by the 17 genotypes of Cortibel, Século XXI and Paluma. This cluster was subdivided into six groups (Figure 4): CLM, CIX, CRG, CXII, CXI, CBLG, CBRM, CV, Paluma, CRM, SXXI, CIII, CLG and CXVI (group 1); CSLG (group 2); CX and CXIII (group 3); CXVII and CVII (group 4); Roxa and Pedro Sato (group 5); and Sassaoka (group 6).

According to the heatmap, overall the genotypes had 5-mC values lower than 25%. The cultivars Roxa and Pedro Sato differed from this pattern, with values higher than 25% during the post-pruning phase, whereas the cultivar Sassaoka presented high values at all phases and the highest value for the post-pruning phase. The first and largest group consisted of 14 genotypes, of which eight were commercial, presenting mean values of 5-mC between 15% and 25%, and maintaining a certain homogeneity across the development phases. With the exception of the cultivars Paluma and CXVI, all other genotypes from this group did not show significant differences among development phases.

The CSLG cultivar had a divergent pattern, with a low value of %5-mC for post-pruning and subsequent increase and maintenance of this constant value. The genotypes CX and CXIII differed from the others by their hypomethylation condition (lower value of %5-mC) during post-harvest. The genotypes CXVII and CVII formed another group, with a low value of %5-mC during the harvest phase.

There was no statistically significant variation in global 5-mC considering different vegetative and reproductive organs (i.e. young and developed leaves, branch, apical bud and floral bud) in five genotypes (Figure 5). The percentage of 5-mC among tissues ranged from 22.26% to 23.20%. For the apical bud, young leaf and developed leaf, values of 22.41%, 22.45% and 22.55% 5-mC were found respectively, without significant differences between tissues.

Figure 4. Heatmap depicting the percentage of 5-mC for each genotype during the five development phases throughout the production cycle: The darker the color, the higher the %5-mC. Genotype abbreviations and descriptions are provided in Table 1.

Figure 5. Percentage of 5-mC in vegetative and reproductive organs according to HPLC. DL: developed leaf; YL: young leaf; AB: apical bud; B: branch; FB: floral bud; ns: not significant. Paluma and Século XXI did not present floral bud during collection. Genotype abbreviations and descriptions are provided in Table 1.

DISCUSSION

The mean global DNA methylation, measured by %5-mC, varied according to the assessed guava genotype as well as the stage of the production cycle, considering the vegetative and reproductive phases. While DNA methylation remains constant in some genotypes from post-pruning to the post-harvest phase, the occurrence of methylation and demethylation processes can be detected in other genotypes. In Cortibel X, for instance, an increase in %5-mC occurs from pruning to flowering, indicating the occurrence of DNA methylation mechanisms in this period, whereas after flowering until post-harvest there is a decrease in %5-mC, indicating the occurrence of demethylation mechanisms. On the other hand, for the genotypes Paluma, Pedro Sato and Roxa, DNA methylation decreases from post-pruning to flowering and then remains constant until the post-pruning phase.

In this light, the phenotypic variance (traditionally divided into genotype and environmental variance) shown by these genotypes in previous studies (Coser et al., 2012; Silva, 2017a, b) is also influenced by epigenetic variation (Tsaftaris, 2008). This variation occurs due to mechanisms based on chromatin and DNA methylation, which result in a semi-independent epigenetic inheritance system that connects genetic and environmental control (Richards, 2006).

Epigenetic variation among genotypes, which was attested in guava genotypes for the first time in this study, may have implications for plant breeding, given that inheritable variation within populations is the basis for selection and genetic improvement. It is now known that, in addition to mutations that create genetic variation implicit to phenotypic characteristics, epimutations represent a new source of variation for selection (Tsaftaris, 2008). For the selection of the genotypes in question, it is important to consider that some genotypes have little to no variation in the studied epigenetic characteristic of cytosine methylation during the development phases, whereas others vary significantly. The epigenetic variation seen in the distinct plant may constitute an important resource for plant breeding in non-model species with regard to the identification of traits of agronomic interest. After all, the inheritance of these variations is a necessary condition, contributing to the epibreeding of the crop (Latutrie et al., 2019).

Epialleles are variations in genes, caused by methylation, that can be inherited (Kalisz and Purugganan, 2004). The importance of methylated epialleles in plant breeding requires determining the variation in methylation patterns among the genotypes of the population; the degree to which methylation patterns affect phenotypes; and how much epigenetic variations linked to higher phenotypes are reliably inherited (Tsaftaris, 2008; Gallusci et al., 2017). The latter factor is favored in plants derived from clonal propagation, such as guava, since the transmission of epigenetic marks seems to be more stable through mitosis than meiosis (Gallusci et al., 2017; Latutrie et al., 2019).

The cultivar Sassaoka, which showed the highest %5-mC, was one of the most productive cultivars in the experimental orchard evaluated in Mimoso do Sul – ES (Silva, 2017b). Yield is a trait known to be affected by many genes, while also suffering high environmental influence. In the case of guava, yield is further influenced by pruning, which modifies the phenological phases of the plant, allowing for harvests to be scheduled outside of the normal season, increasing productivity (Singh et al., 2015). The cultivar Sassaoka showed a %5-mC peak during the post-pruning phase, followed by a reduction in its value and constancy during other phases. This indicates that the cultivar is heavily influenced by

pruning and responds to this process with an increase in %5-mC level, which can contribute to its high productivity.

The genotypes Roxa and Pedro Sato presented the second highest value of %5-mC and the same pattern of high %5-mC during the post-pruning period, with subsequent reduction and maintenance of this value. However, these cultivars did not present yield as high as Sassaoka in the field, and the cultivar Roxa had the lowest production among the evaluated genotypes (Silva, 2017b). This demonstrates that although pruning is an important technique in guava production and stimulates methylation processes, yield will depend on plant genotype as well as on epigenetic control.

The post-pruning and post-harvest stages were the ones with the greatest differences among genotypes for %5-mC level. Cases of hypo- and hypermethylation were observed during the post-pruning phase, as well as cases of hypomethylation during the post-harvest phase. Therefore, the stress that occurred in these phases alters the expression of responsive genes through DNA methylation (Kim et al., 2010; Luo et al., 2012). Other abiotic stresses, such as saline, osmotic and cold stress, have been reported as hypo- or hypermethylation inducers depending on type of stress, species, and even on level of stress (Labra et al., 2004; Pavet et al., 2006; Choi and Sano, 2007). Hypo- and hypermethylation phenomena were shown to occur according to the guava genotype evaluated in this study. However, it is important to consider that, also depending on the genotype, the degree of pruning and harvesting is different, since they depend on the vegetative structure of the plant and the number of fruits produced.

The most significant changes in %5-mC among the genotypes occurred during the progression from vegetative to reproductive phase, and they may be related to epigenetic patterns that act in the reproductive process of the plant. There were, however, different behaviors in this phase transition. Some genotypes increased in %5-mC, others decreased, and others had no significant change. This diversity of behaviors may be related to the fact that the genotypes started their transition between phases at different times. Floral buds generally began to appear between 75 and 90 days after pruning. However, for the genotypes CVIII, CXII and Século XXI their emergence occurred sooner, at 15 days after pruning, with at least one of the replicates (Silva, 2017a). These genotypes presented stable behavior regarding DNA methylation between the phases evaluated.

The interaction between the genotypes and the stages of development observed in this study was similar to what was found by Silva (2017a), who demonstrated that the genotype plays a large role in the reproductive and vegetative phases of guava trees. In 12 genotypes, the methylation level did not change across development phases. Among these genotypes are CLG, CLM, CBRM, CV, CIII and CRG, which presented better general adaptability in experiments performed at two cultivation sites and during four harvests between 2014 and 2016; and Cortibel LM and Cortibel RM, which were the ones with the best performance in the face of environmental variations (Silva, 2017b). On the other hand, the genotypes Cortibel SLG, Paluma and Sassaoka, which presented %5-mC alterations during their development phases, are among those with the best performance in favorable environments, i.e., having adaptability specific to favorable environments (Silva, 2017b). This adaptability may be derived from the genetic constitution of the genotypes, and indicates that the genotypes with the highest adaptability have their %5-mC less affected by cultural treatment and development stages, while those with lower adaptability are more sensitive to environmental variations, responding with epigenetic changes.

The genotypes analyzed in this study were previously evaluated by other authors with regard to morphological, quantitative, molecular, productive and essential oil characteristics (Coser et al., 2012; Mendes et al., 2017; Silva, 2017a; Silva, 2017b; Souza et al., 2018). In most groupings, a large number of Cortibel genotypes were clustered, as observed in this study. As for the clustering based on the composition of essential oils, a %5-mC pattern very similar to that obtained in this study was observed. In addition to the formation of a large group containing most Cortibel genotypes, the genotypes CXIII and CX were separated and grouped; CVII and CXVII in some cases were also grouped; the cultivars Paluma and Século XXI remained in the same group; Pedro Sato and Sassaoka were grouped in some cases; and Roxa was shown to be different from the others. This suggests that there may also be an influence of epigenetic mechanisms on the composition of guava essential oils. The relationship between the overall DNA methylation profile and variation in caryophyllene composition reported in different studies with essential oils (Mendes et al., 2017; Souza et al., 2018) suggests that these characteristics are negatively correlated; in this study, the genotypes that presented the lowest %5-mC, CX and CXIII, also presented the highest levels of (E)-caryophyllene, the main constituent of essential oils from these guava genotypes (Mendes et al., 2017; Souza et al., 2018).

There was no difference in %5-mC among the assessed tissues. Because the analysis took place after the genotypes were fully developed as well as in intense production phase, this may indicate that the 5-mC content remained stable among them. Indeed, considering the long-lasting stable transmission of gene expression states over generations, such expression does tend to remain unchanged between tissues (Springer, 2013).

DNA methylation plays a greater role in the differentiation among genotypes than between development phases. In addition, although many changes in gene expression occur, and particularly during tissue development in plants, there is little evidence associated with %5-mC tissue-specific variation in this development process. In a study carried out in different tissues of maize, there were few variations in the levels of DNA methylation that could provide regulatory mechanisms of development associated with the gene expression in the culture (Eichten et al., 2013).

CONCLUSIONS

The percentage of global 5-methylcytosine in the guava genome is variable, depending on the genotype. Some genotypes showed higher stability in this parameter, with no variation throughout the production cycle, while others presented significant variations between the vegetative and reproductive periods. The most hypomethylated genome was seen during the post-harvest vegetative period in genotypes that have genetic proximity reported in previous studies (CX and CXIII), demonstrating that genetic characteristics also influence the epigenetic profile.

The highest variation in overall percentage of 5-methylcytosine occurred during the post-pruning period, which is characterized by intense vegetative activity after drastic pruning for production, according to the recommendations for the crop. The Sassaoka genotype presented the highest hypermethylation values, associated with differing behaviors in the production cycle related to agronomic traits, constituting an important marker for breeding studies. There were no differences in methylation between vegetative and reproductive tissues in the adult guava plants.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING STATEMENT

All data is provided in the supplementary material, and the genotypes were defined in the Material and Methods section.

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