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ARTICLE

Impact of Nitrogen Fertilization on Nutrient Content of 'Paluma' Guavas During Fruit Development: *A Target Metabolite Approach*

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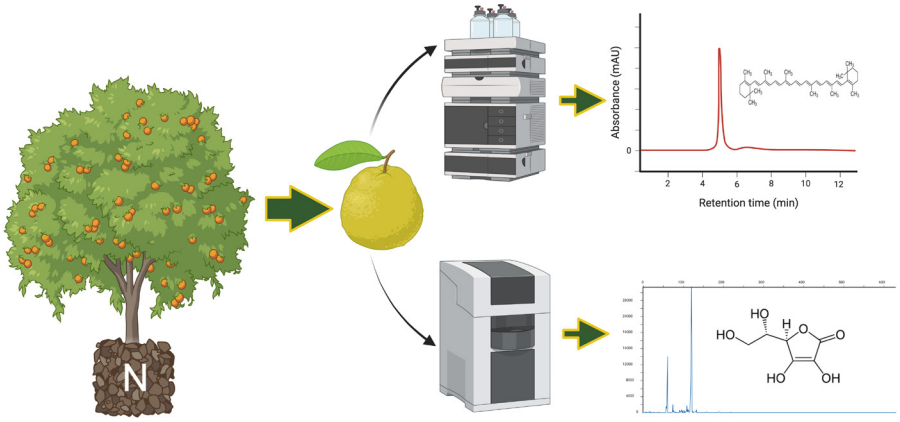

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Guava (*Psidium guajava* L.) is a tropical fruit with significant economic potential due to its *in natura* and industrialized consumption. This fruit has a high nutritional value due to bioactive substances, such as high levels of vitamins and carotenoids. Our study evaluated the impact of varying levels of nitrogen fertilization on the development of guava fruits over a set of targeted metabolites. For this purpose, guava plants were fertilized with 500, 1000, and 2000 g N_2/tree, and their fruits were evaluated at four stages of development. In all stages, the diameter of the fruits was measured, and the pH and soluble solids were analyzed. Capillary electrophoresis was used to determine the concentrations of neutral sugars and ascorbic acid. High-performance liquid chromatography was used for β-carotene analysis. The study showed that nitrogen fertilization did not change fruit size, but the highest concentration of fertilizer (2000 g N_2/tree) reduced the concentrations of ascorbic acid and β-carotene. From these results it can be concluded that the intermediate fertilization level

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(1000 g N_2/ tree) produced most of the metabolite levels studied, in contrast to the highest nitrogen level applied, which showed similar behavior to the absence of fertilization. Correct nitrogen fertilization plays a fundamental role in producing high-quality fruits, influencing the content of various metabolites produced by the fruits. Therefore, adjusting fertilization practices according to the specific needs of their crops helps produce valuable fruits. Meanwhile, incorrect nitrogen fertilization generates direct costs for farmers regarding lost production and imposes significant costs on the environment and society.

Keywords: *Psidium guajava*, metabolites, fruit development, nitrogen fertilization, nutritional value

INTRODUCTION

Guava is a tropical fruit that belongs to the Myrtaceae family, *Psidium* genus, and *Psidium guajava* L. specimen. Brazil is one of the world's largest and most essential guava producers in South America.[1] Different red pulp varieties exist, such as Ogawa, Pedro Sato, Cascuda de Pariquera-Açu, and Iwao, apart from the Paluma, Rica, and Século 21 cultivars.[2] 'Paluma' is one of the most widespread cultivars since it has excellent characteristics for fresh and industrial consumption.

The guava fruit pleases the consumer both *in natura* and in industrialized forms, like juices, jams, and in addition to other products. Due to this diversity, guava is economically essential for local and exportation markets. Its value is directly related to the pulp color (white or red), the size, and the quality of the fruit, which is indicated by the total titratable acidity (TTA), the total soluble solids contents (SSC), their ratio (SSC/TTA), the soluble sugars, and vitamins.[3]

Guava has outstanding nutritional values, rich in vitamins and bioactive substances, such as ascorbic acid (vitamin C),[4,5] and high levels of carotenoids,[6,7] which confers nutraceutical properties and allows for classifying it as a functional food. These characteristics, however, may change under the influence of different factors, including the life cycle of the fruit and fertilization practices.[7,8]

The guava fruit goes through various stages during its development after germination. Five facts stand out: i) size and weight increase due to the cell multiplication and biosynthesis of novel compounds; ii) maturation, when biochemical, physiological, and structural changes occur, is marked by an increase in the sweetness, softening, and color change by the decrease of chlorophyll and the development of carotenoid pigments and anthocyanins; iii) physiological maturity, stage at which the harvest of the fruit occurs; iv) ripening, the stage when there is no more fruit growth, is just metabolic maintenance with the accumulated substrates, and v) senescence, stage in which there is tissue aging and cell death.[9]

Few studies in Brazil correlate fertilization levels as having increased production without affecting fruit quality. Because of this, the fertilization of guava is usually done empirically, in contrast with most economically relevant fruits.[10] According to Natale et al.,[11] guavas are highly responsive to soil fertility. However, increased production at the expense of rising levels of fertilizer can cause a reduction in fruit quality. For example, the decrease in fruit quality can relate to a variation in internal and external color, which is related to chlorophyll and carotenoid contents, while the total soluble solids are directly related to soluble sugars. Thus, using a targeted metabolomics approach, the objective of this work was to establish analytical methods to evaluate the influence of nitrogen fertilization on 'Paluma' guava trees based on essential bioactive metabolites of the fruit, such as neutral sugars, ascorbic acid (AA), and β-carotene, throughout the four different stages of fruit development.

MATERIALS AND METHODS

Chemicals and apparatus

A Milli-Q water purification system (Millipore Corporation, Bedford, MA) produced the purified water used in all solutions. A variety of suppliers provided the chemicals used in this work: sodium hydroxide (purity ≥ 98%), potassium phosphate dibasic anhydrous (purity ≥ 99%), HPLC grade acetone (purity ≥ 99%), and methanol (purity ≥ 99%) were from Synth (São Paulo, Brazil), while sodium phosphate salts - dibasic and monobasic (purity ≥ 99%) were from Mallinckrodt (St. Louis, MO). Other chemicals such as

EDTA - ethylenediaminetetraacetic acid (purity ≥ 99%), phosphoric acid (purity ≥ 99%), and petroleum ether (purity ≥ 95%) were from Quemis (Joinville, Brazil), oxalic acid (purity ≥ 99%) was from Suprapur (Merck Millipore Brazil), and the HPLC grade acetonitrile (purity ≥ 99%) was from Panreac (Barcelona, Spain). We used a pachymeter (Mitutoyo, Kawasaki, Japan) to measure the fruit diameters. The pH and total soluble solids were measured using a pH meter (Digimed, Piracicaba, Brazil) and a refractometer (Abbe – 2WAJ), respectively.

Sampling

The guava fruits were provided by Prof. Dr. Willian Natale (Department of Soils and Fertilizers – São Paulo State University, Jaboticabal, SP, Brazil). They were collected from *Indústria de Polpas e Conservas VAL* Ltda's orchard in Vista Alegre do Alto, SP, Brazil. The soil of the planting area is dystrophic Argisol Red-Yellow,[12,13] and the climate, according to the Köppen climate classification,[12–14] is the Cwa subtropical, with short, moderate, dry winters and hot, rainy summers.

Sample preparation and storage

The sampling of the fruits occurred in four periods between May and July 2010, representing four different stages of the fruit development: Collection #1 (C1) was in May, three months after flowering; Collection #2 (C2) occurred during the first fortnight of June; Collection #3 (C3) was at the second half of June; and Collection #4 (C4) occurred in July, five months after flowering. At each one of these stages, the samples collected were fertilized with different doses of nitrogen (urea, 45% N) coded as N0 (no nitrogen), N1 (500 g per tree), N2 (1000 g per tree), and N3 (2000 g per tree). Prof. Natale's group previously determined the ripening stages of the guava fruits and the nitrogen concentrations applied in previous studies.[13,15,16] After each collection, fruits were sorted, identified, and packed in plastic bags, and all samples were stored at -18 °C until processed.

Analysis of physical and chemical parameters

The first parameter to evaluate the fruit's development is its diameter. We measured the fruits' longitudinal (LD) and transversal (TD) diameters to calculate the ratio LD/TD. The pH and the total soluble solids (TSS - expressed as °Brix index) were measured directly from crushed guava juice without dilution.

Analysis of the major neutral sugars and ascorbic acid by capillary electrophoresis (CE)

Extraction of sugars and ascorbic acid

The extraction of sugars was achieved by homogenizing guava fruits with water for 30 min under magnetic stirring, followed by filtration to retain the pulp.[17] The extraction and analysis of ascorbic acid, the determination of total ascorbic acid (AAT), and dehydroascorbic acid (DIA) were performed according to Liao[18] using oxalic acid as a substitute for metaphosphoric acid.

Instrumentation and operating parameters

Sugars – the major neutral sugars were separated, identified, and quantified using P/ACE MDQ capillary electrophoresis equipment (Beckman-Coulter). The neutral sugars were analyzed according to the method described by Rovio and colleagues,[19] using a fused silica capillary (75 μm i.d.; 57 cm total and 50 cm effective length), hydrodynamic injection at 0.5 psi for 3 s, following the injection of the electrolyte solution at the same conditions, and direct detection at 270 nm. The capillary temperature was set at 15 °C and the separation voltage at +14 kV. The running electrolyte solution was prepared by mixing 450 mmol L^{-1} stock solution of $Na_2HPO_4.2H_2O$ with 1 M NaOH to the final concentration of 36 mmol L^{-1} and 130 mmol L^{-1}, respectively, yielding a buffer solution at pH 12.8. The capillary conditioning prior to each analysis consisted of 1 mol L^{-1} NaOH for 15 min, 0.1 mol L^{-1} NaOH for 5 min, Milli-Q water for 5 min, and then electrolyte for 10 min. Between the replicates, the capillary conditioning was 0.1 mol L^{-1} NaOH for 5 min, Milli-Q water for 5 min, and the electrolyte for 5 min. Running electrolyte and NaOH solution were

filtered through a 0.45 µm membrane syringe filter before use. The quantification was based on analytical calibration curves prepared from dilutions of the stock standard solution (5 mg mL^{-1} glucose, fructose and sucrose) in water, ranging from 0.05 to 0.55 mg mL^{-1}.

Ascorbic acid – the analysis conditions for ascorbic acid by CE differed slightly from those for sugars. The fused silica capillary was the same (75 µm i.d.; 57 cm total and 50 cm effective length), but the hydrodynamic injection at 0.5 psi was more prolonged (5 s), and the detection was at 265 nm. The capillary temperature was higher (25 °C), while the separation voltage was the same (+14 kV). The running electrolyte solution was prepared by mixing a 20 mmol L^{-1} stock of dibasic sodium phosphate with monobasic sodium phosphate, pH 8.0. The capillary conditioning was slightly different from the sugar analysis and was carried out with 1 mol L^{-1} NaOH for 10 min, 0.1 mol L^{-1} NaOH for 2 min, Mili-Q water for 1 min, and the electrolyte for 3 min. The capillary was rinsed with the electrolyte between the replicates for 10 min. Running electrolyte and NaOH solution were filtered through a 0.45 µm membrane syringe filter before use.[18]

The identification of sugars and ascorbic acid was performed by comparing the migration times with standard solutions and confirmed by spiking them to the samples. The quantification was based on analytical calibration curves prepared from dilutions of the stock standard solution (1 mg mL^{-1} ascorbic acid in 1 mmol L^{-1} EDTA / 2% oxalic acid), ranging from 2 to 120 µg mL^{-1}.

Analysis of β-carotene by high-performance liquid chromatography (HPLC)

Extraction of β-carotene

β-Carotene was extracted in acetone, followed by partitioning with petroleum ether. According to Rodriguez-Amaya, the extracts followed evaporation (30–35 °C) to decrease the volume and then kept under nitrogen gas until dry.[20] Before the analyses, the samples were resuspended in 2 mL acetone and filtered on a 0.45 µm filter.

Instrumentation and operating parameters

Analyses were performed on liquid chromatography equipment with diode array detection (DAD) (Shimadzu) using a reversed-phase column (C18, ODS-2, 150 × 4.6 mm, 3 µm) at room temperature. A scan from 200 to 700 nm was used to record the UV-Vis spectrum, and the monitoring of β-carotene was fixed at 450 nm. The mobile phase was prepared by mixing HPLC grade methanol (≥ 99.9%), ethyl acetate (≥ 99.9%), and acetonitrile (≥ 99.9%) in the ratio 1:1:8 (v/v/v). The column was equilibrated for 20 min daily, and the analysis sequence was carried out at a mobile phase flow rate of 1 mL min^{-1} in isocratic elution mode.[21]

The identification of β-carotene in the samples was achieved by comparing its elution time with the standard and its UV-Vis spectrum obtained by the diode array detector (λ = 450 nm) with the literature results.[21] We used an analytical calibration curve prepared from dilutions of the stock standard solution (2000 µg mL^{-1}) in acetone for quantification purposes, ranging from 80 to 800 µg mL^{-1}.

Statistical and Principal Component Analysis (PCA)

All the collected data were subjected to ANOVA analysis,[22] with 95% significance (0,05), followed by Tukey's test to verify significant differences between each variable and the ripening process at different nitrogen fertilization levels. The statistical results in this study are represented as mean value +/- standard deviation (SD). Relative standard deviations (RSDs) are calculated as the ratio between the SD value and its corresponding mean in percentage. Other analytical figures of merit, like the limit of detection (LOD) and limit of quantitation (LOQ), were obtained using (SD x 3)/CS and (SD x 10)/CS, respectively, where SD is the standard deviation, and CS is the slope of the analytical curve, according to ANVISA (Agência Nacional de Vigilância Sanitária - Brazilian regulatory agency).[23] PCA used the quantitative data obtained from each metabolite analysis. The data filled a matrix with 16 lines corresponding to samples and five columns corresponding to the targeted metabolites. The Statistica Analysis Software[24] was used to perform the PCA analysis.

RESULTS AND DISCUSSION

Analysis of the physical and chemical parameters

We used ANOVA to evaluate quantitatively the influence of nitrogen fertilization levels and the different ripening stages on LD, TD, LD/TD, pH, and TSS. Based on the data in Table I, we can see the increase in TD and LD during fruit development for all fertilization levels. Also, we can see that nitrogen fertilization did not significantly affect the fruit size, which is the most valuable feature for commercialization. The fertilization level did not influence the ratio LD/TD; this relation tended to unity during the fruit development, a feature of this cultivar. Nitrogen fertilization, according to the literature, does not affect fruit size, but studies have observed that the application of this nutrient in guava increased flowering and the number of fruits produced by the plants.[13,14] In this context, we can relate nitrogen as a constituent of several essential metabolites for plant cell maintenance, such as amino acids, proteins, and nucleic acids. These metabolites are transported by the phloem vessels in the form of elaborated sap to the vegetative buds of the plants where flowering and fruiting occur; for this process to occur efficiently, the growth of the roots and the vegetative part is stopped.[25] In addition, nitrogen is one of the constituents of the chlorophyll molecule; the deficiency of this compound causes the yellowing of the leaves and, consequently, the reduction of the photosynthetic area that will not produce the sap elaborated in sufficient quantity, which may cause the decline of flowering and fruiting.[25]

In addition to the development of the fruits, we observed a decrease in the pH (Table I) in all fertilization levels. For the ripe guavas, we observed the lowest pH for the fruits with no nitrogen fertilization (N0), which was 3.59. According to Manica,[26] for mature fruits, a pH higher than 3.5 indicates the need to add organic acids in the guava processing food, aiming for better final product quality. Therefore, we observed that an increase in nitrogen fertilization could raise the costs of the final guava products because of the need for acidic additives in the processing. The acidity in guava is due to organic acids, mainly citric acid, present in the vacuoles of cells, in free form or combined with salts, esters, and glycosides. The organic acid content tends to decline (with a consequent rise in the pH of the fruit) because ethylene induces the synthesis of several hydrolytic enzymes that destroy chlorophylls, degrade cell walls, hydrolyze starch, synthesize anthocyanins and carotenoids and reduce organic acids and phenolic compounds as the fruit matures.[25] We had a response contrary to this process: as the fruit ripened, there was a decrease in pH values, as seen in Table I, but still, the final pH was slightly higher than 3.5. No studies were found in the literature that corroborated our results. In addition, Lima et al.[27] observed that excessive nitrogen fertilization can delay fruit ripening but did not observe any change in pH due to high concentrations of organic acids.

Another parameter was the ºBrix, which measures total soluble solids (TSS) dissolved in the guava juice. It was impossible to obtain the guava juice from the first two collections because of the small quantity of water in the greenest fruits. Because of this, the °Brix was limited mainly to the last two collections. For most fertilization levels, we observed an increment of soluble sugars between the two collections, except for the N1 fertilization level (Table I). The total soluble solids (ºBrix) indicate the number of solids that are dissolved in the fruit juice; this parameter is used as a maturity index for certain fruits, including guava, which must have its value between 9 and 10 ºBrix with a minimum of 6 ºBrix in the harvest period. These solids are represented by sugars (glucose, fructose, and sucrose), which in guava range from 51 to 91%, representing a significant nutritional value for the fruits; in addition to sugars, there are organic acids, amino acids, vitamins, and pectins. High levels of ºBrix are desirable for fresh consumption and industrial processing.[28]

Table I. Longitudinal (LD) and transversal (TD) diameters, LD/TD ratio, pH and total soluble solids of guava fruits at different stages of development. Results expressed as [mean +/- SD (RSD %)].

	Collection	Fertilization Level			
		N0 (0 g N_2/tree)	N1 (500 g N_2/tree)	N2 (1000 g N_2/tree)	N3 (2000 g N_2/tree)
LD (cm)	C1	4.35^{aA} ± 0.34 (7.81)	4.67^{aA} ± 0.23 (4.92)	4.71^{aA} ± 0.60 (12.7)	4.43^{aA} ± 0.59 (13.3)
	C2	4.37^{aA} ± 0.15 (3.43)	4.62^{aA} ± 0.38 (8.22)	4.79^{bA} ± 0.37 (7.72)	4.69^{aA} ± 0.22 (4.69)
	C3	6.81^{aB} ± 0.59 (8.66)	6.67^{aB} ± 0.15 (2.25)	5.90^{bB} ± 0.29 (4.91)	6.66^{aB} ± 0.75 (11.3)
	C4	7.27^{aB} ± 0.88 (12.1)	8.02^{aC} ± 0.71 (8.85)	8.16^{aC} ± 0.77 (9.44)	8.18^{aC} ± 0.67 (8.29)
TD (cm)	C1	3.58^{aA} ± 0.18 (5.03)	3.61^{aA} ± 0.22 (6.09)	3.55^{aA} ± 0.10 (2.82)	3.63^{aA} ± 0.21 (5.78)
	C2	3.69^{aA} ± 0.15 (4.06)	3.67^{aA} ± 0.20 (5.45)	3.83^{aB} ± 0.30 (7.83)	3.75^{aA} ± 0.38 (10.1)
	C3	5.18^{aB} ± 0.42 (8.11)	5.03^{abB} ± 0.37 (7.36)	4.73^{bC} ± 0.23 (4.86)	5.29^{aB} ± 0.46 (8.69)
	C4	7.35^{aC} ± 0.83 (11.3)	7.08^{aC} ± 0.34 (4.80)	7.13^{aD} ± 0.31 (4.35)	7.13^{aC} ± 0.35 (4.91)
LD/TD	C1	1.22^{aAB} ± 0.07 (5.74)	1.29^{aA} ± 0.06 (4.65)	1.33^{aA} ± 0.17 (12.8)	1.22^{aA} ± 0.15 (12.3)
	C2	1.19^{aA} ± 0.08 (6.72)	1.26^{aA} ± 0.06 (4.76)	1.26^{aA} ± 0.09 (7.14)	1.26^{aA} ± 0.08 (6.35)
	C3	1.31^{aB} ± 0.11 (8.40)	1.33^{aA} ± 0.09 (6.77)	1.25^{aA} ± 0.05 (4.00)	1.26^{aA} ± 0.10 (7.94)
	C4	0.99^{aC} ± 0.13 (13.1)	1.13^{aB} ± 0.07 (6.19)	1.14^{aA} ± 0.06 (5.26)	1.15^{aA} ± 0.13 (11.3)
pH	C1	6.43^{aA} ± 0.01 (0.16)	4.62^{bA} ± 0.01 (0.21)	6.22^{cA} ± 0.01 (0.16)	6.06^{dA} ± 0.01 (0.17)
	C2	4.90^{aB} ± 0.01 (0.20)	4.68^{bB} ± 0.01 (0.21)	5.00^{cB} ± 0.01 (0.20)	5.13^{dB} ± 0.01 (0.19)
	C3	3.76^{aC} ± 0.01 (0.27)	3.79^{bC} ± 0.01 (0.26)	4.03^{cC} ± 0.01 (0.25)	3.64^{dC} ± 0.01 (0.27)
	C4	3.59^{aD} ± 0.01 (0.28)	3.86^{bD} ± 0.01 (0.26)	3.83^{cD} ± 0.01 (0.26)	3.94^{dD} ± 0.01 (0.25)
°Brix (Solid Soluble Content)	C1	—	—	—	—
	C2	—	—	8.00 ± 0.50 (6.25)	—
	C3	9.50^{aA} ± 0.50 (5.26)	8.50^{bcA} ± 0.50 (5.88)	9.00^{abA} ± 0.50 (5.56)	8.00^{cA} ± 0.50 (6.25)
	C4	9.55^{aA} ± 0.50 (5.24)	8.50^{aA} ± 0.50 (5.88)	9.55^{aA} ± 0.50 (5.23)	9.50^{aA} ± 0.50 (5.26)

For each data set (LD, TD, LD/TD, pH, and °Brix): average values followed by the same letter (a, b, c, and d) in the line did not differ between each other with the different levels of nitrogen fertilization by ANOVA test, with a 95% significance (0.05); average values followed by the same letter (A, B, C, and D) in the column did not differ between each other with the stage of development by the ANOVA test, with a 95% significance (0.05).

Neutral sugars

Figure 1 shows the electropherograms of the patterns of sucrose, glucose, and fructose sugars, all at a concentration of 0.1 mg mL^{-1} (Figure 1a), with a sample of guava from collection 4, fertilized with N_2 (Figure 1b). The experimental conditions of this analysis are described in the Materials and Methods section.

The limits of detection (LOD) and quantification (LOQ) of sucrose were 0.068 and 0.228 mg 100 g^{-1}; glucose 0.052 and 0.174 mg 100 g^{-1}; and fructose 0.046 and 0.150 mg 100 g^{-1}, respectively. The repeatability for sucrose was 1.38% (migration time) / 3.00% (peak area), for glucose was 1.50% (migration time) / 4.00% (peak area) and for fructose was 1.54% (migration time) / 9.00% (peak area). The reproducibility for sucrose was: 3.38% (migration time) / 14.0% (peak area), for glucose was 1.16% (migration time) / 16.0% (peak area) and for fructose was 1.27% (migration time) / 24.0% (peak area).

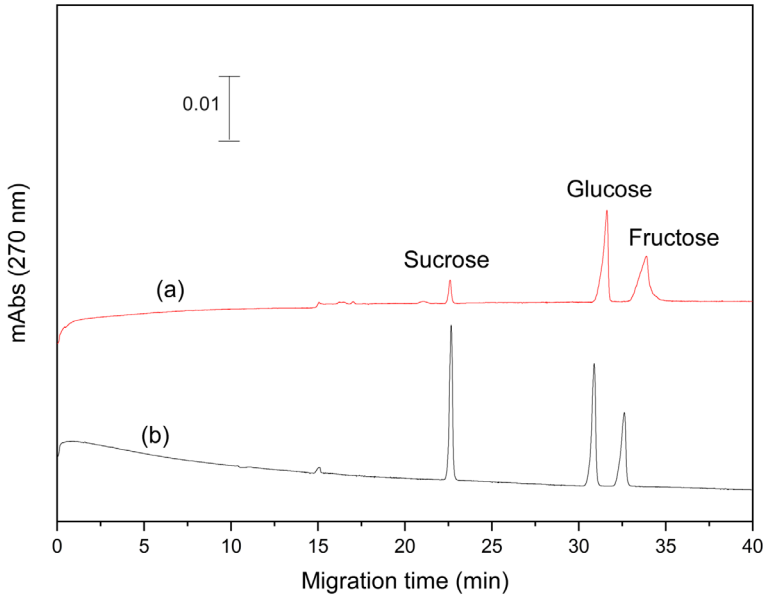


Figure 1. Electropherogram showing the separation of three neutral sugars (sucrose, glucose and fructose) by capillary electrophoresis. (a) the neutral sugars present in guava (sample N2 – C4) and (b) a mixture of neutral sugar standards at a concentration of 0.1 mg mL^{-1}.

There is no determination in the literature of the concentrations of sucrose, fructose, and glucose in guava pulp analyzed by capillary electrophoresis or using any other analytical technique for this matter, so we could not compare the limits of detection and quantification found in our study. Generally, we find in the literature the analysis of total sugars that, according to the normative instruction of the Brazilian Ministry of Agriculture,[28] the values of total sugars in guava should be at least 700 mg 100 g^{-1} of pulp, which agrees with the values of sugars analyzed in this study when we add their concentrations, as can be observed in Table II. Other studies have shown this relationship between nitrogen fertilization and the increase in these sugars, such as that carried out by Lima et al., who observed an improvement in fruit quality, including an increase in soluble solids and soluble sugars, especially in the advanced ripening stages of guava fruits.[27]

Table II shows the behavior of the reducing sugars, glucose, and fructose, as well as the non-reducing sugar sucrose, during the development of the guava tree under different amounts of nitrogen applied to the guava trees. These sugars are responsible for the sweet taste of fruits, with fructose being the most abundant sugar in guava,[29] where it is observed that the increase in sweetness in the fruit is related to the formation and continuous increase in fructose levels.[29]

In general, the concentrations of reducing sugars showed a significant increase, as seen in Table II, in the stages of development of guava, collections C1 and C2, with collections C3 and C4 within the same fertilization category. When nitrogen applications are related to the fruit development stages (the collections), it is observed that nitrogen fertilization influenced the concentrations of reducing sugars since the guavas that did not receive nitrogen fertilization (N0) differed significantly in the glucose and fructose concentrations of the collections that received the fertilizer, except for the C3 and C4 collections of the N3 fertilization, which according to ANOVA presented similar results to N0.

Regarding sucrose, within the amounts of nitrogen applied (columns in Table II), it is observed that there was a decrease in the concentrations of this non-reducing sugar throughout the maturation process of the guava fruit, except for N3 fertilization where the values C1 and C4 sucrose are similar; among the collections (lines in Table II) C1 showed a proportional increase in sucrose with the application of nitrogen, as there was a difference between all sucrose concentrations and doses with the doses of nitrogen applied; for C2 the fertilization N1 and N2 differed from N0, for C3 only the doses N1 and N3 differed from N0, and for C4 there was a difference between the values of N0 and the others, with N2 and N3 being similar each other.

Some studies have reported a less pronounced increase in sucrose for some cultivars relative to fructose and glucose.[30] This fact may occur because, during some stages of maturation, glucose and fructose originate from the degradation of sucrose and the reserve of polysaccharides; as the fruits develop, the sucrose content decreases simultaneously with an increase in reducing sugars. Furthermore, according to Jain et al.[31] the increase in these sugars is related to the increase in the degree of sweetness during aging, providing adequate characteristics for consumption. After this step, the content of reduced sugars decreases since the metabolism of the fruits starts to use them as a source of energy.

When glucose and fructose are correlated with sucrose in guava fruits, it is observed that within the fertilization (lines of Table II), C1 did not show a significant difference between the concentrations of sugars as for N1 and N2 fertilizations higher, and similar values were observed for glucose and fructose, but not for sucrose, which was not detected in samples fertilized with N1. According to the literature,[8,32] an increase in the content of neutral sugars is expected with fruit development. During maturation, since the beginning of fruit development, neutral sugars are present in the form of starch (reserve substance) and pectin, which are used for energy production and biosynthesis of organic compounds, among others.[25]

No report in the literature mentions the relationship between the sugar content of the fruit and the concentration of nitrogen fertilization. However, it is known that sucrose, a secondary product of photosynthesis, can be transported from the leaves after production to the organs for consumption as the fruits develop. Furthermore, photosynthesis directly depends on nitrogen, as chlorophyll is a molecule rich in this compound.[25]

Table II. Mean concentrations of soluble sugars in guava fruits at different stages of development with varying levels of nitrogen fertilization. Results expressed as mean value +/- SD (RSD%).

Soluble sugars	Collection	Fertilization Levels			
		N0 (0 g N_2/tree)	N1 (500 g N_2/tree)	N2 (1000 g N_2/tree)	N3 (2000 g N_2/tree)
Sucrose (mg 100 g^{-1})	C1	195^{aA} ± 16 (8.2)	153^{bA} ± 7 (4.6)	109^{cA} ± 7 (6.4)	215^{dA} ± 10 (4.6)
	C2	261^{aA} ± 93 (35.6)	87^{bB} ± 1 (1.1)	136^{bcA} ± 1 (0.7)	180^{acAB} ± 54 (30.0)
	C3	69^{aB} ± 8 (11.6)	21^{bC} ± 1 (4.8)	52^{aC} ± 11 (21.2)	136^{cB} ± 25 (18.4)
	C4	83^{aB} ± 5 (6.)	0^{bD} ± 0	275^{cD} ± 47 (17.1)	261^{cA} ± 43 (16.5)
Glucose (mg 100 g^{-1})	C1	162^{aA} ± 11 (6.8)	232^{bA} ± 23 (9.9)	184^{bA} ±17 (9.2)	260^{cA} ± 13 (5.0)
	C2	529^{aA} ± 216 (40.8)	240^{bA} ± 7 (2.9)	190^{bB} ± 4 (2.1)	130^{bA} ± 5 (3.9)
	C3	793^{aB} ± 393 (49.6)	163^{bA} ± 36 (22.1)	3180^{cC} ± 274 (8.6)	888^{aB} ± 548 (61.7)
	C4	1137^{aB} ± 277 (24.4)	2831^{bB} ± 771 (27.2)	2857^{bC} ± 954 (33.4)	1451^{aC} ± 143 (9.9)
Fructose (mg 100 g^{-1})	C1	207^{abA} ± 18 (8.7)	191^{aA} ± 7 (3.7)	215^{bA} ± 11 (5.1)	344^{cA} ± 17 (4.9)
	C2	605^{aAB} ± 232 (38.4)	296^{bA} ± 3 (1.0)	248^{bA} ± 10 (4.0)	176^{bA} ± 24 (13.6)
	C3	790^{aB} ± 491 (62.2)	418^{aA} ± 305 (72.9)	2906^{bB} ± 223 (7.7)	854^{aB} ± 377 (44.2)
	C4	994^{B} ± 289 (29.1)	2659^{aB} ± 1391 (52.3)	2673^{aB} ± 1391 (52.0)	1110^{aB} ± 307 (27.7)

Means followed by the same letter (a, b, c, and d) on the line do not differ concerning fertilization by ANOVA, with a 95% significance level (0.05). Means followed by the same letter (A, B, C, and D) in the column do not differ from the developmental stages using ANOVA with a 95% significance level (0.05).

Ascorbic Acid

Ascorbic acid (AA) is considered an essential antioxidant in plant tissue, as it acts in its development as a cofactor of several enzymatic reactions and protects plant cells against the action of error,[33] in addition to being a modulator in the plant cell signaling, including cell division and expansion, and cell wall formation during plant growth.[34] In fruits, the AA content varies according to the species, variety, growing conditions, stage of development, and harvest point. AA also depends on the photosynthetic process, ambient temperature, and sun exposure.[35]

Due to the low concentrations of and dehydroascorbic acid (DIA) (Table III), it was possible to determine these metabolites only in C4 of N0 and in C3 and C4 of fertilization N1, N2, and N3 because in C1 and C2 of N1, N2 and N3, and C1, C2, and C3 of N0, the concentrations of these compounds were below the detection and quantification limits of the method used.

Table III. Mean concentrations of ascorbic acid (AA), total ascorbic acid (Total AA), and dehydroascorbic acid (DIA) in guava fruits at various stages of development (C1, C2, C3, and C4) under different nitrogen fertilization levels. Results are expressed as mean value +/- SD (RSD %).

	Collection	Fertilization Levels			
		N0 (0 g N_2/tree)	**N1 (500 g N_2/tree)**	**N2 (1000 g N_2/tree)**	**N3 (2000 g N_2/tree)**
AA (mg 100 g^{-1})	**C1**	nd	nd	nd	nd
	C2	nd	nd	nd	nd
	C3	nd	16^{aA} ± 4 (25.0)	24^{bA} ± 6 (25.0)	12^{aA} ± 1 (8.3)
	C4	22^{aA} ± 1 (4.5)	21^{aA} ± 4 (19.0)	36^{bA} ± 3 (8.3)	29^{cB} ± 6 (20.7)
Total AA (mg 100 g^{-1})	**C1**	nd	nd	nd	nd
	C2	nd	nd	nd	nd
	C3	nd	30^{aA} ± 7 (23.3)	35^{aA} ± 9 (25.7)	13^{bA} ± 1 (7.7)
	C4	30^{aA} ± 4 (13.3)	41^{abA} ± 4 (9.8)	64^{cB} ± 6 (9.4)	54^{abcB} ± 14 (25.9)
DIA (mg 100 g^{-1})	**C1**	nd	nd	nd	nd
	C2	nd	nd	nd	nd
	C3	nd	13^{bA} ± 3 (23.1)	11^{bA} ± 3 (27.3)	1.11^{aA} ± 1.4
	C4	7^{aA} ± 4 (57.1)	20^{aA} ± 7 (35.0)	29^{aB} ± 9 (31.0)	25^{aB} ± 9 (36.0)

'nd' denotes values not detected. Means followed by the same letter (a, b, c, and d) on the line do not differ concerning fertilization by ANOVA, with a 95% significance level (0.05). Means followed by the same letter (A, B, C, and D) in the column do not differ regarding developmental stages using ANOVA with a 95% significance level (0.05).

Figure 2 shows an electropherogram comparing the ascorbic acid extracted from the guava sample from the C4 collection, the N2 fertilization level (Figure 2a), and the ascorbic acid standard concentration of 20 µg mL^{-1} (Figure 2b). The experimental conditions of this analysis are described in the Materials and Methods section.

The limits of detection and quantification of AA were 2.22 and 7.40 mg 100 g^{-1}, respectively. The total AA and DIA values were calculated according to the AA concentration found. The repeatability was 0.16% (migration time) / 5.00% (peak area) and the reproducibility was 3.47% (migration time) / 13.0% (peak area).

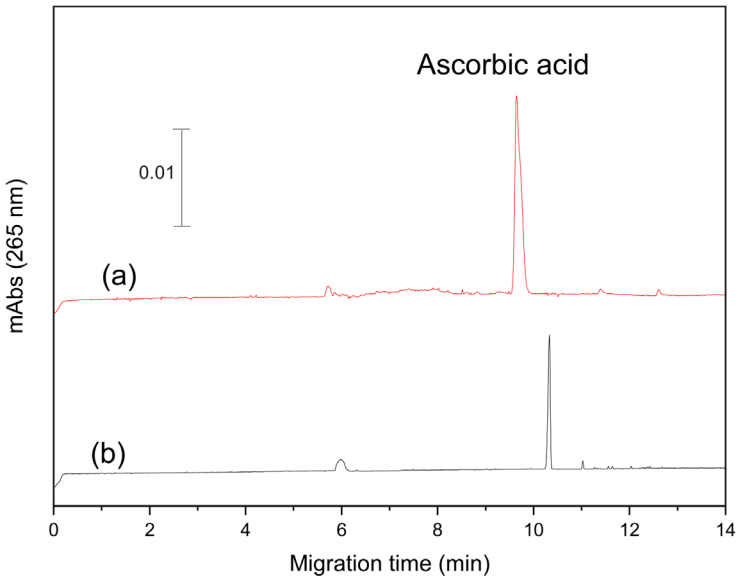


Figure 2. Electropherogram depicting the capillary electrophoresis analysis of ascorbic acid. (a) ascorbic acid present in the guava sample (N2 – C4) and (b) an ascorbic acid standard at a concentration of 20 µg mL^{-1}.

According to Gomez and Lajolo[36] and Dube & Singh,[8] there is an increase in the total content of vitamin C during the development of guava, indicating that the synthesis of ascorbic acid prevails when the need for its action as an antioxidant is maximum in the fruit. Dube & Singh[8] also showed that the total ascorbic acid content of the apple cultivar decreased after 150 days of fruit development. Furthermore, studies on other cultivars have observed this increase during fruit development and ripening,[32,37] of which were found in the literature, in which cultivar Paluma presented 58.74 mg of ascorbic acid for 100 g of pulp.[38]

The dehydroascorbic acid that results from the AA oxidation process represents 10% of the total AA of vegetables, which tends to increase with the storage period. However, the content of this acid also varies according to the fruit, vegetable, and the period of its senescence.[39,40]

Among the values of AA, total AA, and DIA detected, it was observed that for AA, the concentrations of C3 were similar between N1 and N3 and differed for N2; in C4, they were similar for N0 and N1 and different for N2 and N3. For total AA, only C3/N3 showed a significant difference, and within the C4 collection, only N2 fertilization had a significant result, according to ANOVA. The DIA, only C3/N3, proved to be different from the other values found, being the lowest DIA value measured (1.11 mg 100 g^{-1}), and for C4, there was no significant difference between the values obtained. The best values of AA, total AA, and DIA were found for N2 fertilization (29.66 to 63.95 mg 100 g^{-1}), demonstrating that fertilization with high nitrogen levels (N3) does not favor the production of AA.

Lima et al. (2008)[27] evaluated the variation of fertilization in the stages of maturation. They observed that in the green guava, there was a decrease in AA with an increase in fertilization. The same behavior was observed in the mature guava only when maximum fertilization was used. Some authors observed that nitrogen fertilization reduced AA levels in grape, tangerine, and orange juice[41] and in papaya, there was no significant variation in AA levels.

β-carotene

Beta-carotene and lycopene are natural plant compounds responsible for the colouring of some fruits and vegetables, but more than just colouring food, these nutrients have important functions in the body, especially in the human skin.[20,31,42]

According to the literature, chlorophyll is concomitantly reduced with the increase in carotenoids, which can be visually observed in colors from green to yellow.[20,31,42] In general, for different fruits, there is an increase in the content of total carotenoids in the mature stage about the initial stages, confirmed by various authors who evaluated β-carotene during the development of guava.[8,20,30] Carotenoids are found in all photosynthetic organisms; they are constituents of thylakoid membranes and are associated with the proteins of the photosynthetic apparatus. The relationship between chlorophyll and carotenoids lies in when the carotenoids absorb light and transfer it to chlorophyll; hence, carotenoids are called accessory pigments.[25]

Figure 3 shows the HPLC chromatograms obtained for the guava samples collected at the C4 stage, fertilized with N2 (Figure 3a), and the β-carotene standard of the 200 μg L^{-1} (Figure 3b). The experimental conditions of this analysis are described in the Materials and Methods section.

The limits of detection and quantification of β-carotene by HPLC-UV were 0.668 and 2.23 mg 100 g^{-1}, respectively. The reproducibility was 3.10% (migration time) / 6.00% (peak area).

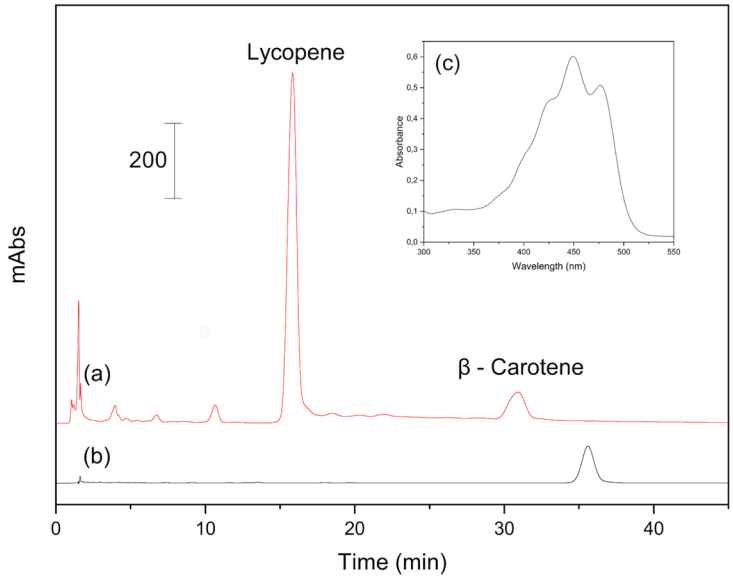


Figure 3. High-performance liquid chromatography (HPLC) chromatograms of (a) β-carotene in the guava sample (N2 – C4) and (b) β-carotene standard. (c) β-carotene UV spectrum purity.

The β-carotene concentrations (Table IV) were below the detection and quantification limits of the method for all forms of nitrogen fertilization in collection 1. Evaluating the C2 about the applied amounts of nitrogen, a significant difference was found between the values of β-carotene, with the highest values found in N0, the concentrations decreased until N2, and its value increased again in N3. In Collections 3 and 4, the behavior is similar, with the lowest values in N0 and increasing until N2, when they decreased again in N3. The highest values of β-carotene were found in C4 - N2, as seen in Table IV. Observing the data in Table IV, it can be concluded that nitrogen fertilization (N3) caused a decline in β-carotene levels, presenting values close to those found for samples without fertilization (N0).

Evaluating the amounts of nitrogen applied (columns in Table IV), the plants that did not receive nitrogen (N0), N1, and N3 showed the highest concentrations of β-carotene in C2 and C3, the lowest analyte value. In C4, they increased the concentration of β-carotene again. With N2 fertilization, the β-carotene values gradually increased from C2 to C4, reaching the highest levels in the study.

No literature was found relating β-carotene with nitrogen application, but the variations of this compound found in this work may be associated with the incidence of light and the temperature of the period in which

the fruits were grown, as these are parameters that affect the variation of β-carotene in plants.[29,35,37,43] The light incidence rapidly degrades β-carotene; it was observed that at 30 min of exposure, the concentration of β-carotene reduces to half of the initial concentration; another physical factor observed was that the increase in temperature causes a similar effect to that of light.[29,35,37,43]

According to some authors, β-carotene is the second most predominant carotenoid in mature guavas.[6] We observed a high content of this carotenoid in the analyzed guavas of the cultivar Paluma. The values of mature fruits (C4 at N1 and N2 levels) were higher than those observed for guavas from Israel, which were 4960 mg 100 g^{-1},[42] from Indonesia (984 mg 100 g^{-1}),[39] and from the Brazilian guava cv. Palmyra ICA-1 (155 mg 100 g^{-1}).[43]

Table IV. Concentration of β-carotene in guava (mean ± SD (RSD %)) during the fruits' development in different fertilization levels

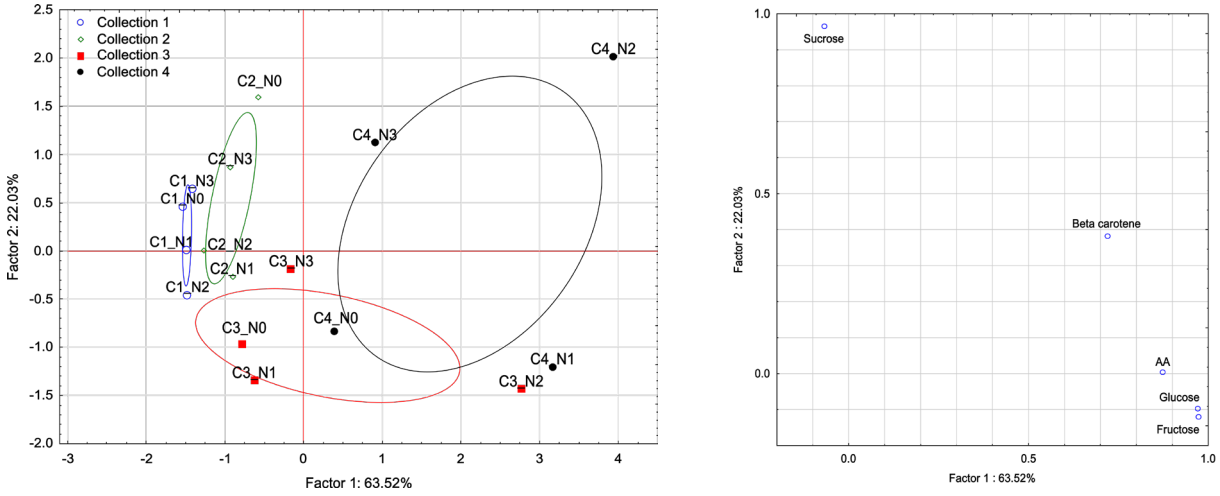
	Collection	Fertilization Levels			
		N0 (0 g N_2/tree)	N1 (500 g N_2/tree)	N2 (1000 g N_2/tree)	N3 (2000 g N_2/tree)
β-Carotene (µg 100 g^{-1})	C1	-	-	-	-
	C2	7797^{aA} ± 28 (0.36)	6789^{bA} ± 40 (0.59)	2815^{cA} ± 463 ((16.45)	8616^{dA} ± 76 (0.88)
	C3	699^{aB} ± 29 (4.15)	1138^{bB} ± 93 (8.17)	3270^{cA} ± 108 (3.30)	1852^{dB} ± 19 (1.03)
	C4	1107^{aC} ± 2 (0.18)	14247^{bC} ± 274 (1.92)	18691^{cC} ± 330 (1.76)	2705^{dC} ± 784 (28.98)

Means followed by the same letter (a, b, c, and d) on the line do not differ concerning fertilization by ANOVA, with a 95% significance level (0.05). Means followed by the same letter (A and B) in the column do not differ regarding developmental stages using ANOVA with a 95% significance level (0.05).

Principal Component Analysis (PCA)

PCA evaluated the correlation of the targeted metabolites from guava fruits collected during four stages and four nitrogen fertilization levels. Figure 4 shows the scores (a) and loadings plots (b) obtained by PCA. The scores graph represents the identification of patterns and groupings between samples. The principal component 1 (Factor 1) is responsible for 63.52% of the total variance in the data. Therefore, most of the information in the data can be represented along this axis, while the second principal component (Factor 2) accounts for another 22.03% of the total variance. When we sum up PC1 and PC2, we can observe 85.55% of the total variance of the data, which indicates that these components are sufficient to provide an excellent two-dimensional representation of the multivariate data. As a result, it allows us to visualize and precisely interpret the main differences and patterns between the samples. In this way, we could simplify their complexity without losing significant information, facilitating the interpretation and analysis of the results, Figure 4.

The loadings graph provides information about how each variable contributes to the main components identified in the PCA. Each variable represents its position about the axes of the main components PC1 and PC2, indicating its contribution to each component.



(a) (b)

Figure 4. Scores (a) and Loadings (b) plots obtained from PCA for PC1 and PC2 for guava samples collected during different stages of fruit development (collections C1: red; C2: green; C3: blue; C4: orange) and grown under different levels of nitrogen fertilization (N0 = 0 g N_2/tree; N1 = 500 g N_2/tree; N2 = 1000 g N_2/tree; N3 = 2000 g N_2/tree).

Samples collected on the initial stages 1 (C1) and 2 (C2) are grouped on the left side of PC1. The combined analysis of the scores and loadings plots indicates that sucrose is the primary metabolite responsible for forming these groups, independent of the nitrogen fertilization level from the studied metabolites.

According to the PCA, guava samples in initial development stages have high concentrations of sucrose; for this reason, C1 and C2 are grouped; this result is in line with the literature, which shows that guavas gradually increase the concentrations of this sugar throughout its development reaching its maximum in the stage of full maturation. A study evaluated three guava cultivars (Pedro Sato, Paluma, and Sassaoka) at different stages of development and observed that 60 days after flowering, the fruits had low concentrations of sugars and reached their maximum values 120 days after flowering.[44]

Therefore, it occurs because, during the fruit's initial development, fruit plants generally direct their resources to synthesize essential organic compounds such as amino acids and nucleic acids and accumulate water and mineral salts to grow and develop. After this stage, the fruit begins to mature, which is influenced by the increase in photosynthetic activity, and the plant starts to direct more resources to the production of reserve compounds (such as sugars). Most of the samples from collection 3 (C3) are grouped on the left side of PC1, except the sample from level 2 of nitrogen fertilization (N2), which is grouped on the right side of PC1, along with all the samples from collection 4 (C4). Ascorbic acid, glucose, fructose, and β-carotene were the primary metabolites responsible for forming the right-side group according to the observation of scores and loadings plots, *i.e.*, the metabolites most relevant to mature fruits.

β-carotene may have its concentration influenced by nitrogen fertilization, as it is an essential nutrient for synthesizing proteins and chlorophyll. Chlorophyll is responsible for the green color of leaves and fruits; that is, chlorophyll can influence the synthesis of carotenoids in guava fruits.[45,46]

Nitrogen fertilization did not directly influence the separation of this PCA, but we see a more diverse effect as the fruit matures. For example, C4 is the most dispersed group, indicating the relevance of proper nitrogen fertilization to obtain fruits of desired fruit characteristics and nutraceutical value. Therefore, depending on the concentration applied, it can positively or negatively affect the development of fruits in guava and other fruit-producing plants. Excess nitrogen can increase plant biomass production to the detriment of fruit production and affect carbohydrate synthesis; excess N can also increase the activity of enzymes involved in cellular respiration that consume sugars for energy production. Ideal amounts of

nitrogen can positively influence the quality of the fruits and the concentration of sugars, so it is essential to fertilize in adequate concentrations according to the plant's development stage, type of soil, and climatic conditions.

CONCLUSIONS

We generally observed an increase in the targeted metabolites and fruit development. This increase, however, was not observed for sucrose and β-carotene content in the samples without fertilization, and for N1 samples, sucrose showed a drastic decrease with maturation. The fertilization N2 stands out over the others because it provided higher levels of the metabolites studied, indicating a good balance between the cost of production and organoleptic and nutraceutical values. The maximum fertilization level (N3) presented a similar behavior to the samples without fertilization (N0) for more than one metabolite, indicating a possible toxicity. Therefore, the results indicated that the maximum level is not attractive since, besides diminishing metabolite development, it generates higher production costs. This analytical study showed that guava is a good source of these metabolites/nutrients, and it can be useful for producers to evaluate nitrogen fertilizer use.

The statistical analysis provided by PCA demonstrated a clustering of samples collected in all stages of fruit development, with remarkable separation of C1 and C2 from those collected in stages C3 and C4 of maturation. PCA also showed that the influence of nitrogen fertilization on samples collected at any stage is diverse, indicating that as the fruit grows and matures, the fertilization was a dispersive factor, showing the importance of using the proper level, balancing costs, and aggregate values of the production again.

Conflicts of interest

There are no conflicts to declare.

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