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Article

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[1] Federal University of Parana, UFPR, Curitiba, PR, Brazil; e-mail: debora_defensor@outlook.com.
[2] State University of Ponta Grossa, UEPG, Ponta Grossa, PR, Brazil; e-mail: castuepp@uepg.br.
[3] Brazilian Agricultural Research Corporation, Embrapa Florestas, Colombo, PR, Brazil; e-mail: cristiane.helm@embrapa.br; osmir.lavoranti@embrapa.br; ivar.wendling@embrapa.br.
[4] Federal Technological University of Parana, UTFPR, Curitiba, PR, Brazil; e-mail: marcusliz@utfpr.edu.br, amanda_acm@hotmail.com, rafaimoski@hotmail.com.
[*] Correspondence: ivar.wendling@embrapa.br

Abstract: An especially important species in southern Brazil, yerba mate (*Ilex paraguariensis*) is traditionally consumed as tea, *chimarrão* and *tererê*. Yerba mate consumption has been stimulated by scientific discoveries that have identified high concentrations of bioactive compounds and their health benefits. There are no studies or scientific evidence to certify or guarantee stability of these compounds' concentrations during different years in the same plants. We were interested in quantifying caffeine, theobromine, total phenolic compounds and proteins concentrations in leaves of yerba mate genotypes and their stability in the same plants over four consecutive years. Mature leaves from yerba mate genotypes selected on a provenance and progenies trial were collected in August of 2015, 2016, 2017 and 2018. Methylxanthines (caffeine and theobromine), total phenolic compounds and total protein contents were quantified. Our results indicate large variations between genotypes regarding caffeine (0.035 to 2.385 g 100 g^{-1}), theobromine (0.0004 to 1.772 g 100 g^{-1}), total phenolic compounds (7.028 to 9.424 g 100 g^{-1}) and proteins (10.39 to 16.58 g 100 g^{-1}) contents, and also high stability of those compounds over the four evaluated years. A great variation of the studied bioactive compounds in different *Ilex paraguariensis* genotypes was found and its stability over four consecutive years was showed.

Keywords: yerba mate; breeding; methylxanthines; phenolic compunds; HPLC

1. Introduction

Ilex paraguariensis A.St.-Hil. or yerba mate is native to South America and traditionally consumed as a tonic and non-alcoholic stimulant beverage. From the first reports of its consumption by Guarani Indians to current consumption models, yerba mate has played a central role in the socio-economic and cultural evolution of South American people. Mainly consumed in Argentina, Brazil, Paraguay and Uruguay, its consumption has become a cultural expression [1], reaching 10 kg per person per year in some regions [2]. It is a competitive product in terms of price, quality, regulatory compliance and innovation [3], although regional consumption forms do not favor its commercial expansion to expression markets, with significant lower consumption than its competitors *Coffea* spp. in Europe and North America and *Camelia sinensis* in Asia and Europe [4].

The genetic resources of yerba mate are fundamentally important for selecting specific genotypes that can subsidize the industry and emergence of new products, thus directing science and innovation in this market [5]. Focusing on regional consumption, mate breeding programs have been selecting genotypes with characteristics adapted to biotic and abiotic factors and high production of commercial biomass for decades [5,6]. Recently, emphasis has been placed on selecting genotypes

that contain higher concentrations of certain chemical compounds [4,7–10]. Such findings have aroused interest and increased consumption worldwide, mainly because health benefits provided by these compounds [3,4,11).

Consumed among indigenous people of different ethnic groups, yerba mate has been empirically used to treat different pathologies. Recently, scientific proof of its antioxidant, anti-inflammatory, anti-obesity and cardioprotective functions have been reported [2,4,11]. Yerba mate dry leaf biomass composition is quite rich and diverse, including nutrients, minerals and water-soluble vitamins [4]. Its chemical composition contains polyphenols (phenolic acids, flavonoids), alkaloids (methylxanthines, including caffeine, theobromine, theophylline) and terpenes (carotenoids, saponins) [1,2]. Despite its variety of compounds, most research has focused on the caffeine, theobromine and theophylline contents in commercial products of yerba mate [3,4,12] and antioxidants [2,13].

Chemical compounds present in yerba mate are responsible for its stimulant and medicinal properties [2,4,11]. However, the absence of genetic materials selected for this purpose makes the variation in this content one of the limitations of its use, depending strongly on the preparation mode and quantity ingested, but mainly of the raw material used for production [14–16].

There is a gap in studies on the stability of concentrations of these biocompounds during different years in the same plants, and our hypothesis is that this variation is not influenced by the harvest year. Thus, in this study, we evaluated caffeine, theobromine, total phenolic compounds and protein on leaves of yerba mate genotypes for four consecutive years on the same plants to determine the composition and stability of this compounds over time.

2. Materials and Methods

2.1 Plant material and sample preparation

Fifty-five yerba mate genotypes were selected based on their productivity, leaf morphotype and sex, from a provenance and progenies trial installed in Ivaí-PR, Brazil (25°01'S and 50°48'W, 600 m asl, 1500-1600 mm) [5]. This trial began in November 1997 with 156 progenies in 3 x 2 m spacing and full sun conditions [5], and voucher specimens were preserved at the Fernando Cardoso da Silva Herbarium (HFC) with voucher (HFC 10592), in Colombo-PR, Brazil.

In August of 2015, 2016, 2017 and 2018, mature leaves with no injuries were collected throughout the treetops at a height of ~1.6 m. Leaves were stored in "kraft" packages, identified and sent to the Non-Timber Products Technology Laboratory of Embrapa Florestas. Samples were dried in a microwave oven (power 1,500 W, frequency 2,450 MHz) for approximately 4 min, alternating leaves position at 60-second intervals for homogenous drying [17]. Subsequently, leaves were crushed, sieved at 0.5 mm, packed and stored in a freezer (-20 °C).

To determine the content of caffeine, theobromine and total phenolic compounds, aqueous extracts containing 0.1 g of plant material were prepared in 50 mL of ultrapure water type I heated to its boiling temperature (100 °C under pressure of 1 atm). Then, they were homogenized in ultrasound (Ultracleaner 1,400 A) for 30 min, cooled to room temperature and filtered, with volume completed to 100 mL in a volumetric flask, and frozen (-20 °C) [18].

2.2 Determination of methylxanthines

Extracts were thawed and manually homogenized for 30 s. About 2 mL of extracts were filtered in a 0.22 µm membrane with a syringe and holder. After that, an aliquot was transferred to a 1.5 mL amber vial with teflon cap. These samples were injected directly into the chromatograph. Methylxanthines (caffeine and theobromine) were quantified through High Efficiency Liquid Chromatography (HPLC), using Agilent 1260 Infinity model controlled by ChemStation software and equipped with a G1311B quaternary pump, G1329B automatic injector, DAD G4212B detector and FLD G1321B detector available at the Multi-User Laboratory of Environmental Equipment and Analysis of the Federal Technological University of Paraná (LAMEAA-UTFPR). The Acclaim 120 C18 column (Dionex® 2.1x150 mm, 3 m) with an Acclaim C18 2.1 mm, 5 ⊚m guard cartridge was used to

separate compounds. The conditions used to separate compounds from the aqueous extracts (10 µL injection) were: 30 °C with flow of 0.3 mL min-1 of eluent with the mobile phase A (H2O: acetic acid J.T. Baker – 99.5:0.5 v/v) and B (Merck® acetonitrile - 100%). The wavelength used to detect the compound was 280 nm (fixed). The gradient elution program was: 0-8 min (4% B), 8-12 min (4-5% B), 12-30 min (5% B).

The compounds caffeine (1,3,7-trimethylxanthene) and theobromine (3.7-dimethylxane) were quantified using analytical curves obtained with Sigma® standards for caffeine (R^2 = 0.99989) and theobromine (R^2 = 0.99964). The results were expressed in g of compound per 100 gram of dry sample (g 100 g^{-1}). We studied 54 genotypes for caffeine and 55 genotypes for theobromine.

2.3 Determination of total phenolic compounds

Quantification of total phenolic compounds was made according to Folin-Ciocalteau spectrophotometric method (Singleton and Rossi, 1965), with modifications. Briefly, 0.1 mL of extract, 6.0 mL of distilled water and 0.5 mL of Folin-Ciocalteau reagent were added to a volumetric flask and stirred for 1 minute. Afterwards, 2 mL of 15% Na_2CO_3 solution was added and stirred for another 30 seconds. Final volume was adjusted with distilled water to 10 mL. The reaction was kept in dark at room temperature for 2 hours and subsequently absorbances were recorded in a spectrophotometer at 760 nm. Analytical curve was obtained with total phenolic compounds (3,4,5-trihydroxybenzoic acid) between concentrations of 0.25 and 13 mg L^{-1} (R^2 = 0.9988) and the results expressed in mg of gallic acid were equivalent to the total phenolic compounds per gram of dry sample (mg GAE g^{-1}). We studied 55 genotypes for total phenolic compounds.

2.4 Determination of moisture and proteins

The analyses of moisture and total proteins followed the official methods from Association of Official Analytical Chemists International (AOAC 2016). To determine moisture content, we used thermogravimetric method at 105 degrees, up to constant mass and total protein content by the micro-Kjeldahl method, using total nitrogen content multiplied by conversion factor 6.25 to obtain the total protein content. We studied 54 genotypes for protein and moisture content.

2.5 Statistical analysis

Genotypes stability was observed throughout four years based on the existence of interaction with years analyzed by the AMMI (Additive Mail effects and Multiplicative Interaction) procedure. This procedure complemented the deviance analyses, obtained from generalized linear model with Gamma probability function. Initially proposed by Mendel [19], the AMMI method was used to decompose sources of variation of the additive effects for genotypes and years and the multiplicative effects for interaction via main components. This method provides more accurate estimates of genotypic responses and a graphical interpretation of the results through the biplot procedure with scores of the interaction effects for each genotype and year plotted simultaneously [20]. Interpretation is based on the magnitude and signal of coefficients for each main component axis. Low values indicate genotypes and/or years that contribute little or almost nothing to the interaction, demonstrating statistical stability.

Preliminarily, the model of casualized blocks, processed for each year, was adjusted according to the equation:

$$y_{ijk} = \mu + {}^{b}/_{a_{jk}} + g_i + a_j + ga_{ij} + \varepsilon_{ijk}$$

In which, the concentration of Caffeine, Theobromine, Total phenolic compounds, and Proteins of the 'i' genotype in the 'j'-th year and the 'k'-th block (y_{ijk})was estimated by the overall mean (μ) of the concentration, comprising all the genotypes, per block and per year; by the effect of the 'k'-th block in the 'j'-th year (${}^{b}/_{a_{jk}}$); by the effect of the 'i'-th genotype (g_i); by the effect of the 'j'-th year (a_j); by the effect of the interaction of the 'i'-th genotype with the 'j'-th environment (ga_{ij}); added to

the random error (ε_{ijk}). Following this adjustment, the model AMMI was applied in accordance with the analysis of joint variance proposed by Duarte and Vencovsky [21]:

$$Y_{ij} = \mu + g_i + a_j + \sum_{k=1}^{n} \lambda_k \gamma_{ik} \alpha_{jk} + \rho_{ij} + \overline{e_{ij}}$$

In which, the average response of the concentration of Caffeine, Theobromine, Total phenolic compounds, and Proteins of the 'i' genotype in the year 'j', for the purpose of stability, was obtained through the overall mean (μ) of the concentration comprising all the genotypes by year; by the effect of the 'i' genotype (g_i); by the effect of the year 'j'(a_j); by the summation of the product between the singular value (λ_k), the singular vector (γ_{ik}) corresponding to the 'i'-th genotype in the 'k'-th column vector, and the singular vector (α_{jk}) corresponding to the 'j'-th year in the 'k'-th row vector in the matrix of per-year genotype interaction; by the non-controlled trial variance, named as noise (ρ_{ij}) of the classical interaction of the 'i' genotype with the year 'j', and the mean trial error (e_{ij}). The decomposition of the interaction matrix into row and column vectors was performed through the analysis of principal components. The recovery of variation, for the purpose of measuring genotypic stability, was only performed by the deterministic portion of the components most strongly associated with the interaction (rows and columns of the matrix ga_{ij}), discarding the additional residue ρ_{ij}.

The interpretation of the biplot regarding the GxA interaction was carried out by observing the magnitude and sign of genotype and production year scores on the interaction axes. Therefore, low scores (close to zero) are indicative of genotypes that have contributed little or almost nothing to the interaction, characterizing them as stable. The "ward.D2" method minimizes the sum of the squares of the differences in axis scores within the groups formed during hierarchical clustering [22].

3. Results

The joint analysis of variance for Caffeine, Theobromine, Total phenolic compounds, and Proteins (Tables 1, 3, 5 and 7, respectively) revealed significant differences (p<0,01) for genotypes (G), evaluation years (A), and their interaction (GxA). It was observed that, even with only four evaluation years, it was possible to detect differences among them, indicating rather varied conditions for genotype evaluation. In terms of absolute F-statistic values, we noted that the genotype variation source magnitude was much higher for the traits Caffeine and Theobromine, being accountable for the majority of the observed variation. For the traits Total phenolic compounds and Proteins, the magnitude of the year-variation source was higher. It can thus be inferred that the years effects contributed more significantly to the variation of these traits. The interaction results for all the studied traits indicated either the need to identify and select genotypes capable of capturing genetic gains across different yield years or that those are less susceptible to environment variations due to the year. Based on these results, a more detailed study on the significant interaction was conducted, through the analysis of the phenotypic stability of the genotypes, via AMMI. The multiplicative effect of the interaction GxA was diagnosed through the analysis of the main compounds, by decomposing the sum of the squares of the interaction (SQ) in three axes (IPCA), adopting the positive criterion (use of hypothesis tests), through the F-test presented by Cornelius et al. [23].

3.1 Caffeine

Descriptive statistics results show wide genetic variability for caffeine content in yerba mate progenies, with genotype strongly influencing its concentration. First component describes 58.35% of the variability observed in the genotype-environment interaction (Table 1).

Table 1. Variance analysis associated with the breakdown of interaction effect (GxA) for the concentration of Caffeine of the yerba mate genotypes tested across four years of yield.

Variation sources	GL	SQ	F (Pr>F)
Model	56	119.84	
Genotype (G)	(53)	(119.03)	37.87 (<0.0001)

| High Axes	Value	Variance (%) Explained	Cumulative		GL	SQ	Pr>F
				Year (A)	(3)	(0.81)	4.57 (0.0042)
				GxA	159	9.43	
1	5.50	58.35	58.35	IPCA1	(55)	(5.50)	3.04(<0.0001)
2	2.14	22.74	81.10	IPCA2	(53)	(2.15)	1.23 (0.1491)
3	1.78	18.90	100.00	IPCA3	(51)	(1.78)	1.06 (0.3731)
				Mean Error	290	9.57	
				Adjusted Total	215	129.27	

GL: liberty levels; SQ: sum of the square; Pr>F: probability estimate by F-test (Fischer-Snedecor).

Caffeine content varied from 0.0348 to 2.3846 g 100 g^{-1} on different genotypes (Table 2). Similarity observed in distribution of years and genotypes makes it possible to group samples according to caffeine concentration; however, the large number of genotypes generates overlap, especially in areas of interest.

Table 2. Mean genotypic values and analysis of parameter estimates ($\mu + g$) the mean interaction GxE ($\mu + g + ge$) for caffeine concentration of yerba mate genotypes (EC) tested across four consecutive years.

| Genotypes	Means	Chi-Square	Pr > ChiSq	Genotypes	Means	Chi-Square	Pr > ChiSq
EC16	0.0396	0.17	0.6780	EC44	0.0413	0.05	0.8203
EC17	0.0542	0.79	0.3752	EC16	0.0396	0.17	0.6780
EC18	0.0674	2.71	0.0996	EC44	0.0413	0.79	0.3752
EC19	0.0348	1.32	0.2501	EC45	2.3290	30.50	<.0001
EC20	0.5961	27.19	<.0001	EC47	0.1208	11.35	0.0008
EC21	0.4373	25.61	<.0001	EC48	1.7543	30.13	<.0001
EC22	0.0374	0.43	0.5128	EC49	2.2004	30.44	<.0001
EC23	0.3752	24.52	<.0001	EC50	0.0605	1.70	0.1925
EC24	1.0741	29.17	<.0001	EC51	0.0839	5.47	0.0193
EC25	1.5091	29.88	<.0001	EC52	0.0889	6.80	0.0091
EC26	1.5221	29.90	<.0001	EC53	2.3579	30.52	<.0001
EC27	1.5769	29.96	<.0001	EC65	0.3324	23.33	<.0001
EC28	1.0096	27.96	<.0001	EC66	0.4116	25.15	<.0001
EC29	0.0850	5.89	0.0152	EC67	0.0479	0.15	0.7018
EC30	0.0374	0.38	0.5362	EC68	0.7601	28.15	<.0001
EC31	1.1940	29.42	<.0001	EC69	0.8194	28.29	<.0001
EC32	0.8286	28.44	<.0001	EC70	1.5931	29.98	<.0001
EC33	1.0823	29.19	<.0001	EC71	2.1910	30.45	<.0001
EC34	1.1340	29.30	<.0001	EC72	1.2243	29.47	<.0001
EC35	0.2751	22.21	<.0001	EC73	1.6523	30.04	<.0001
EC36	1.1766	29.38	<.0001	EC74	1.1718	29.01	<.0001
EC37	1.9442	30.28	<.0001	EC76	0.0906	7.09	0.0078
EC38	1.6107	29.99	<.0001	EC77	0.6200	27.19	<.0001
EC39	0.7686	28.19	<.0001	EC78	0.0799	5.00	0.0253
EC40	1.7908	30.16	<.0001	EC79	2.3846	30.51	<.0001
EC41	1.4447	29.80	<.0001	EC80	0.7048	27.29	<.0001
EC42	1.1004	29.23	<.0001	EC81	0.0576	1.18	0.2770
EC43	1.3957	29.74	<.0001	EC82	0.0438	2.80	0.0946

The individually examined years tended to contribute more to the GxE interaction than the genotypes (Figure 1). This can be observed by the greater dispersion of points or vectors associated with the years compared to the vector associated with genotypes in the biplot (IPCA1 x IPCA2).
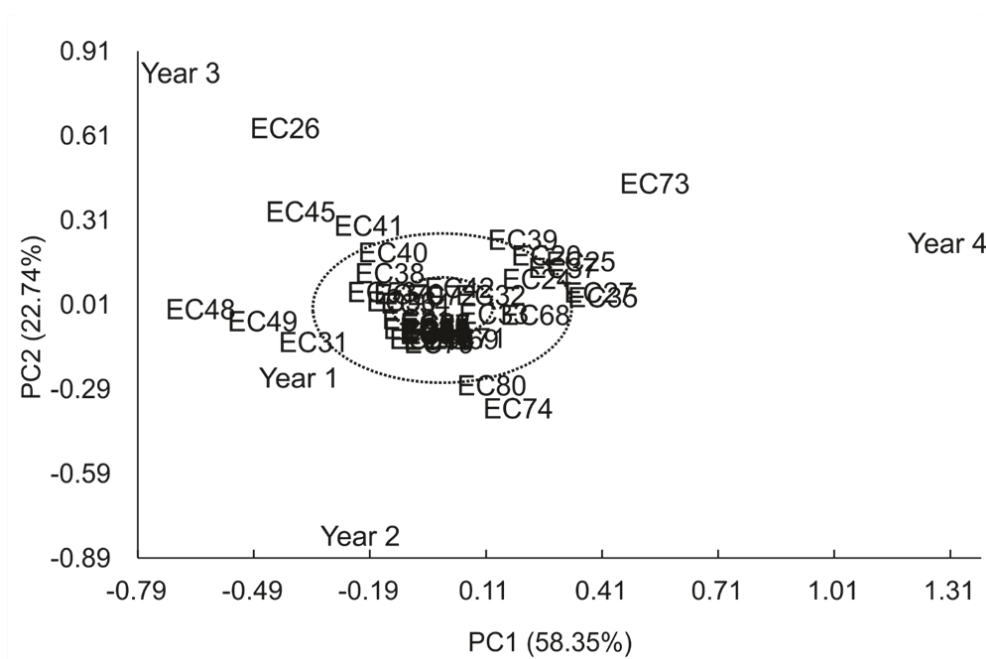
Figure 1. PCA of genotype-environment ratio of the yerba mate genotypes (EC) for caffeine concentration according to PC1 and PC2.

Principal component analysis shows that the first two components describe 81.09% of total variance, implying great dependence of genotype on caffeine concentrations. Horizontal axis accounts for 58.35% of variation, while vertical axis corresponds to 22.74% (Figure 1). Figure 2 shows genotypes stability, with 44,5% of them stable at 1% probability of error (represented in blue). Additionally, 24,1% of genotypes are stable at 5% (represented in green) and 31,5% of genotypes are not stable throughout the four years (represented in red).
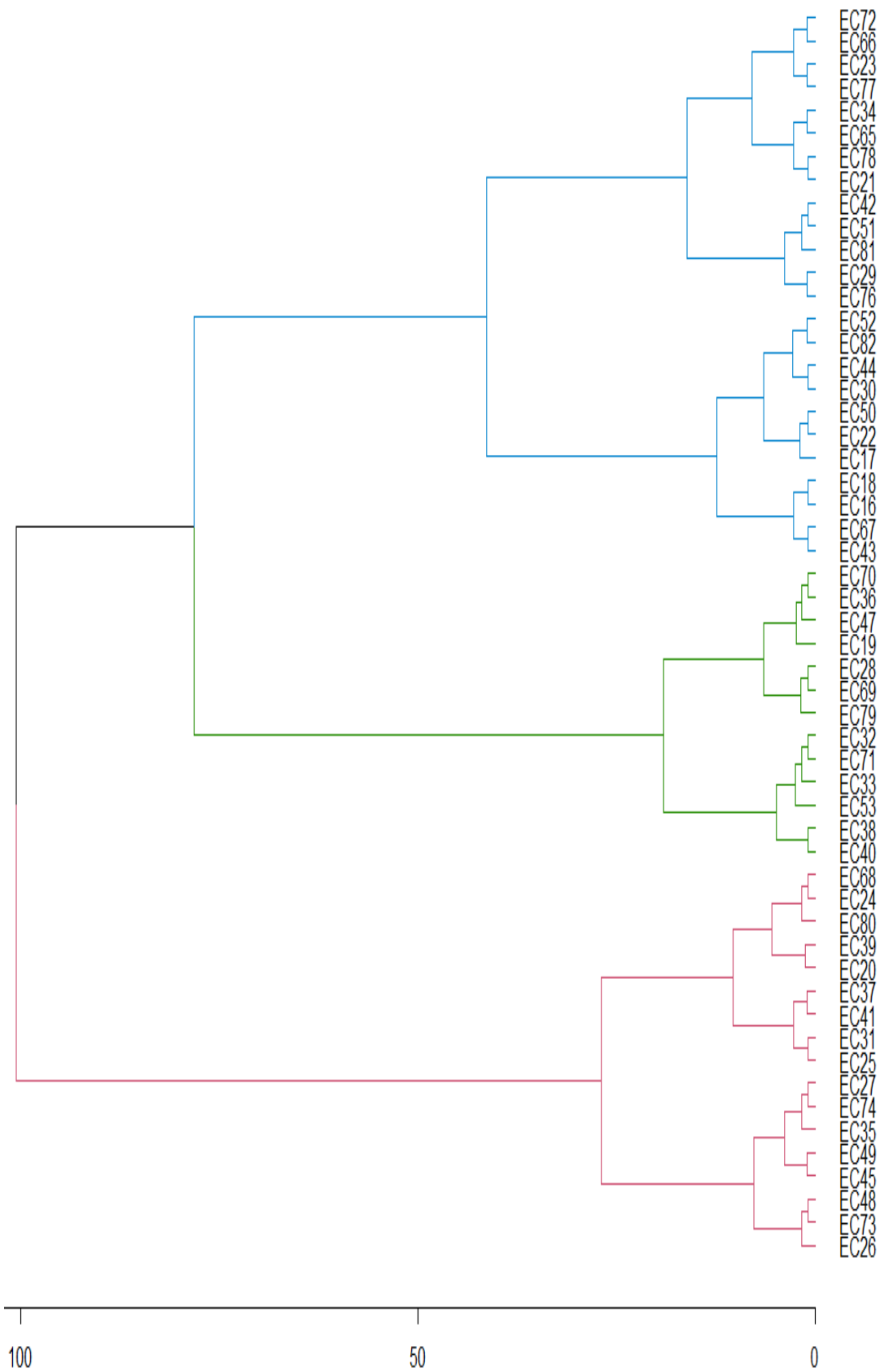
Figure 2. Genotypic stability clustering using Ward's Minimum Variance Method of genotype-environment ratio of yerba mate genotypes (EC) for caffeine concentration according to PC1 and PC2.

3.2 Theobromine

The statistics results show wide genetic variability for theobromine content in yerba mate progenies (Table 3).

Table 3. Variance analysis associated with the breakdown of interaction effect (GxA) for theobromine concentration of yerba mate genotypes (EC) tested across four consecutive years.

	High Axes Value	Variance (%) Explained	Variance (%) Cumulative	Variation sources	GL	SQ	F (Pr>F)
				Model	57	19.86	
				Genotype (G)	(54)	(19.38)	12.71 (<0.0001)
				Year (A)	(3)	(0.48)	5.70 (0.0010)
				GxA	162	4.57	
1	2.73	59.78	59.78	IPCA1	(56)	(2.73)	3.11 (<0.0001)
2	1.25	27.35	87.13	IPCA2	(54)	(1.25)	1.47 (0.0238)
3	0.59	12.87	100.00	IPCA3	(52)	(0.59)	0.72 (0.9237)
				Mean Error	295	4.63	
				Adjusted Total	219	24.43	

GL: liberty levels; SQ: sum of the square; Pr>F: probability estimate by F-test (Fischer-Snedecor).

The theobromine concentrations ranged from 0.0004 to 1.7719 g 100 g^{-1} in the 55 analyzed genotypes (Table 4), with the first component describing 59.78% of variance observed in genotype-environment interaction.

Table 4. Mean genotypic values and analysis of parameter estimates (μ + g) the mean interaction GxE (μ + g + ge) for theobromine concentration in yerba mate genotypes (EC) tested across four consecutive years.

Genotypes	Means	Chi-Square	Pr > ChiSq	Genotypes	Means	Chi-Square	Pr > ChiSq
EC16	0.3026	3.01	0.0829	EC44	0.4760	5.52	0.0188
EC17	0.2586	2.41	0.1204	EC45	0.0161	0.26	0.6073
EC18	0.4678	5.00	0.0253	EC47	0.0627	0.48	0.4885
EC19	0.3431	3.62	0.0570	EC48	0.0076	2.37	0.1240
EC20	0.0447	5.73	0.0167	EC49	0.0898	1.44	0.2300
EC21	0.0862	0.13	0.7216	EC50	0.0543	0.35	0.5519
EC22	0.0294	0.66	0.4177	EC51	0.1211	4.58	0.0323
EC23	0.0688	5.76	0.0164	EC52	0.6187	6.47	0.0110
EC24	0.1058	6.02	0.0141	EC53	0.0173	0.00	0.9627
EC25	0.3101	0.02	0.8859	EC65	0.0237	1.81	0.1786
EC26	0.0200	1.61	0.2045	EC66	0.5809	3.59	0.0582
EC27	0.0303	0.09	0.7673	EC67	0.0045	4.99	0.0255
EC28	0.0112	3.38	0.0661	EC68	0.1182	8.03	0.0046
EC29	0.0373	2.14	0.1431	EC69	0.2487	1.92	0.1657
EC30	0.0004	7.20	0.0073	EC70	0.0162	6.18	0.0129
EC31	1.7719	5.65	0.0174	EC71	0.1529	5.22	0.0224
EC32	0.0493	6.62	0.0101	EC72	0.0796	2.19	0.1390
EC33	0.0081	4.61	0.0317	EC73	0.0581	0.47	0.4911
EC34	0.0739	0.00	1.0000	EC74	0.1612	1.15	0.2838
EC35	0.0199	7.56	0.0060	EC75	0.4280	0.01	0.9351

EC36	0.0230	3.14	0.0766	EC76	0.3440	5.87	0.0154
EC37	0.5567	6.63	0.0101	EC77	0.0090	3.85	0.0499
EC38	0.0997	0.81	0.3695	EC78	0.5312	5.58	0.0181
EC39	0.1649	1.28	0.2572	EC79	0.5640	0.29	0.5904
EC40	0.0709	0.24	0.6236	EC80	0.1005	3.04	0.0814
EC41	0.9768	5.01	0.0251	EC81	0.2100	1.31	0.2528
EC42	0.1173	6.80	0.0091	EC82	0.1230	4.58	0.0323
EC43	0.0602	0.88	0.3491				

Homogeneity of genotype distribution from 0 and 0.5 g 100 g^{-1} shows lower theobromine concentrations in yerba mate plants when compared to caffeine (Figure 3).
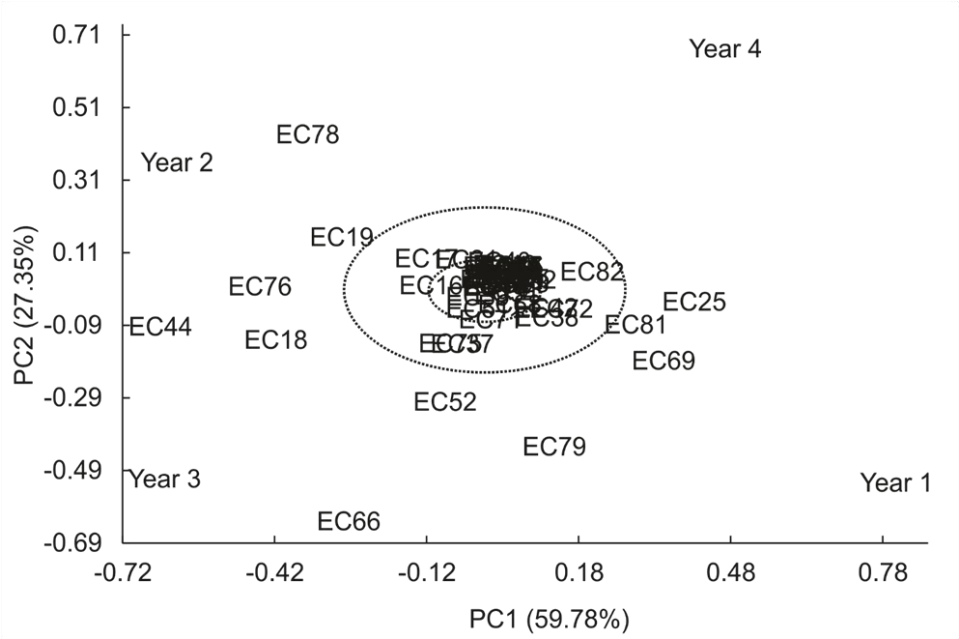
Figure 3. PCA of the first component for theobromine concentration in yerba mate genotypes (EC) in 2015, 2016, 2017 and 2018.

Principal component analysis shows that first two components describe 81.12% of total variance, implying great dependence of genotype on theobromine concentrations. Figure 4 shows the stability of genotypes, with 69.1% of genotypes stable at 1% probability of error (represented in blue). Additionally, 10.9% of the genotypes are stable at 5% (represented in green), while 20% of genotypes are non-stable (represented in red).
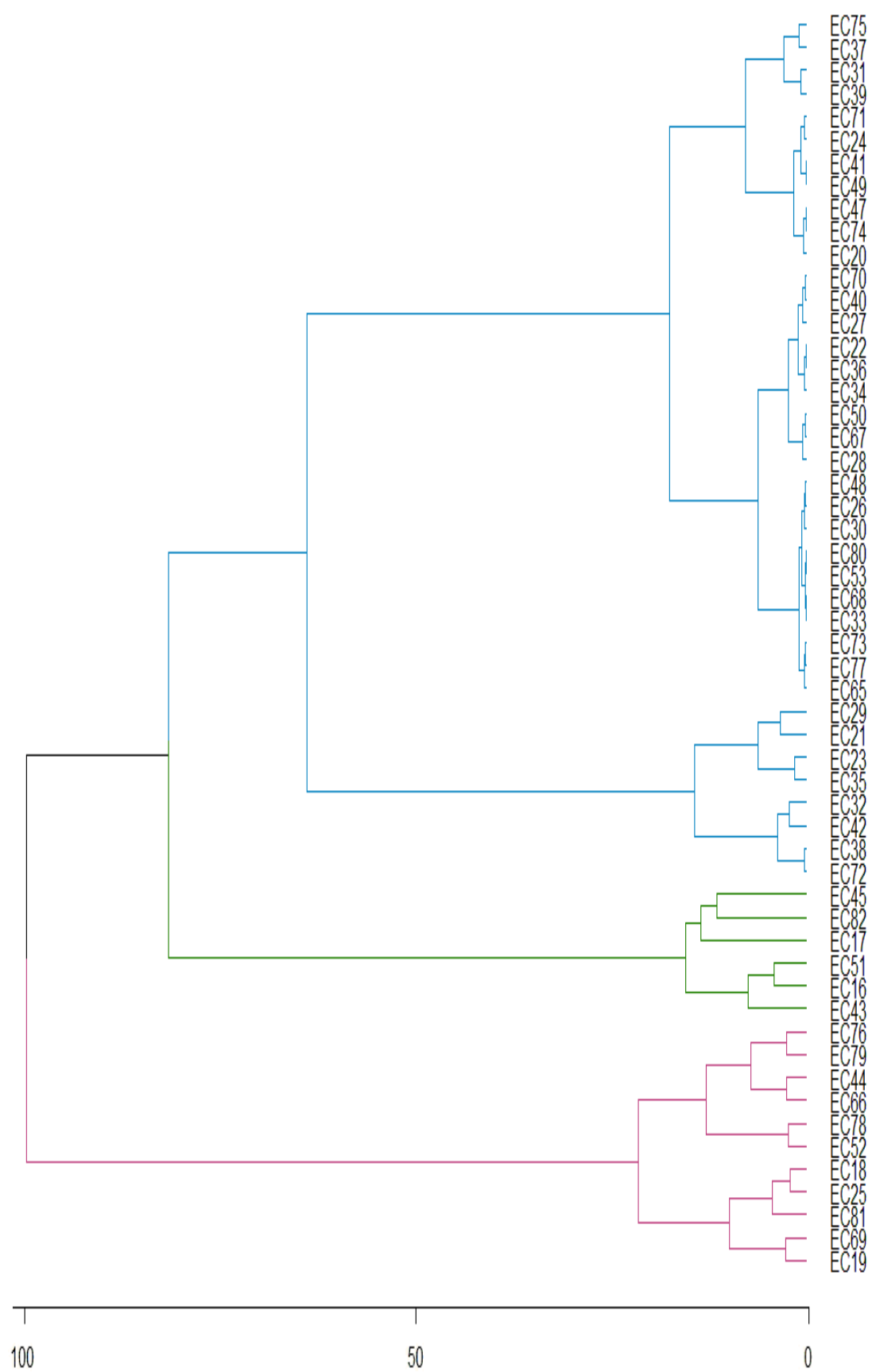
Figure 4. PCA of genotype-environment relationships of the yerba mate genotypes (EC) for theobromine concentration according to PC1 and PC2.

3.3 Total phenolic compounds

Similar to methylxanthines, we found wide variability in total phenolic compounds (Table 5).

Table 5. Variance analysis associated with the breakdown of interaction effect (GxA) for total phenolic compounds concentration of yerba mate genotypes (EC) tested across four consecutive years.

			Variation sources	GL	SQ	F (Pr>F)
			Model	57	95.58	
			Genotype (G)	(54)	(54.76)	1.38 (0.0642)
			Year (A)	(3)	(40.82)	18.50 (<0.0001)
High	Variance (%)		GxA	162	118.50	
Axes Value	Explained	Cumulative				
1	54.03	45.60	45.60	IPCA1	(56)	(54.03)	2.37 (<0.0001)
2	50.41	42.54	88.13	IPCA2	(54)	(50.41)	2.29 (<0.0001)
3	14,06	11.87	100.00	IPCA3	(52)	(14.06)	0.66 (0.9625)
			Mean Error	295	120.12	
			Adjusted Total	219	214.08	

GL: liberty levels; SQ: sum of the square; Pr>F: probability estimate by F-test (Fischer-Snedecor).

The total phenolic compounds ranged from 7.028 to 9.424 g 100 g^{-1} in the 55 genotypes (Table 6). First component describes 45.60% of variability observed in genotype-environment interaction.

Table 6. Mean genotypic values and analysis of parameter estimates (μ + g) the mean interaction GxE (μ + g + ge) for total phenolic compounds in yerba mate genotypes (EC) tested across four consecutive years.

Genotypes	Means	Chi-Square	Pr > ChiSq	Genotypes	Means	Chi-Square	Pr > ChiSq
EC16	9.0128	0.00	0.9479	EC44	8.1318	0.46	0.4999
EC17	8.1033	1.05	0.3049	EC45	8.6207	0.04	0.8496
EC18	7.5834	2.91	0.0882	EC47	8.1529	0.41	0.5245
EC19	**9.2024**	1.33	0.2481	EC48	8.2046	0.29	0.5871
EC20	8.6407	0.28	0.5950	EC49	8.5733	0.01	0.9140
EC21	7.5239	3.31	0.0687	EC50	8.6158	0.03	0.8563
EC22	8.7405	0.16	0.6936	EC51	8.3449	0.09	0.7704
EC23	8.7693	0.20	0.6580	EC52	8.8932	0.08	0.7752
EC24	9.1320	0.41	0.5245	EC53	8.5883	0.02	0.8935
EC25	8.4304	0.26	0.6081	EC65	8.2999	0.00	0.9828
EC26	8.0816	0.59	0.4435	EC66	8.2536	0.16	0.6896
EC27	8.2580	0.55	0.4582	EC67	8.7437	0.22	0.6425
EC28	8.1072	1.07	0.3010	EC68	8.7821	1.85	0.1739
EC29	7.8740	1.32	0.2498	EC69	7.7633	2.43	0.1194
EC30	8.0060	0.82	0.3653	EC70	7.4366	0.68	0.4084
EC31	8.0287	0.75	0.3879	EC71	9.0001	0.00	0.9637
EC32	8.2664	0.19	0.6659	EC72	8.7127	0.01	0.9114
EC33	8.7675	0.19	0.6603	EC73	8.6298	3.58	0.0586
EC34	9.4237	2.27	0.1323	EC74	7.4875	0.00	0.9658
EC35	8.6304	0.04	0.8365	EC75	8.2633	0.02	0.8873
EC36	8.4143	0.03	0.8656	EC76	8.3533	2.67	0.1023
EC37	8.5419	0.00	0.9572	EC77	7.6202	0.25	0.6157
EC38	8.3793	0.05	0.8172	EC78	8.1388	1.79	0.1806

EC39	8.4205	0.03	0.8742	EC79	7.7742	0.87	0.3509
EC40	8.3520	0.08	0.7800	EC80	7.8665	2.45	0.1177
EC41	7.9771	0.92	0.3377	EC81	7.0278	0.66	0.4170
EC42	7.4232	4.07	0.0436	EC82	8.9908	0.00	0.9828
EC43	7.5776	2.50	0.1138				

In Figure 5, the influence of genotype on the composition of total phenolic compounds is clear in the distribution of progenies along the horizontal axis and demonstrates the importance of improving this characteristic in leaf biomass of yerba mate in future cultivars.
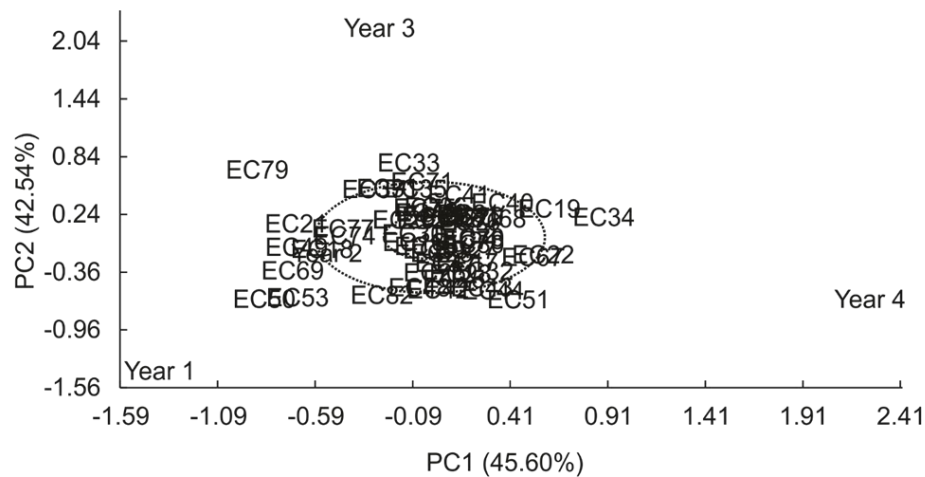
Figure 5. PCA of the first component for total phenolic compounds concentration in yerba mate genotypes (EC) in 2015, 2016, 2017 and 2018.

Principal component analysis for total phenolic compounds concentration shows that first two components describe 88.13% of total variance. The Figure 6 shows the stability of genotypes, with 32.7% of genotypes stable at 1% probability of error (represented in blue), while 36.4% of genotypes are stable at 5% (represented in green) and 30.9% of genotypes are not stable (represented in red).
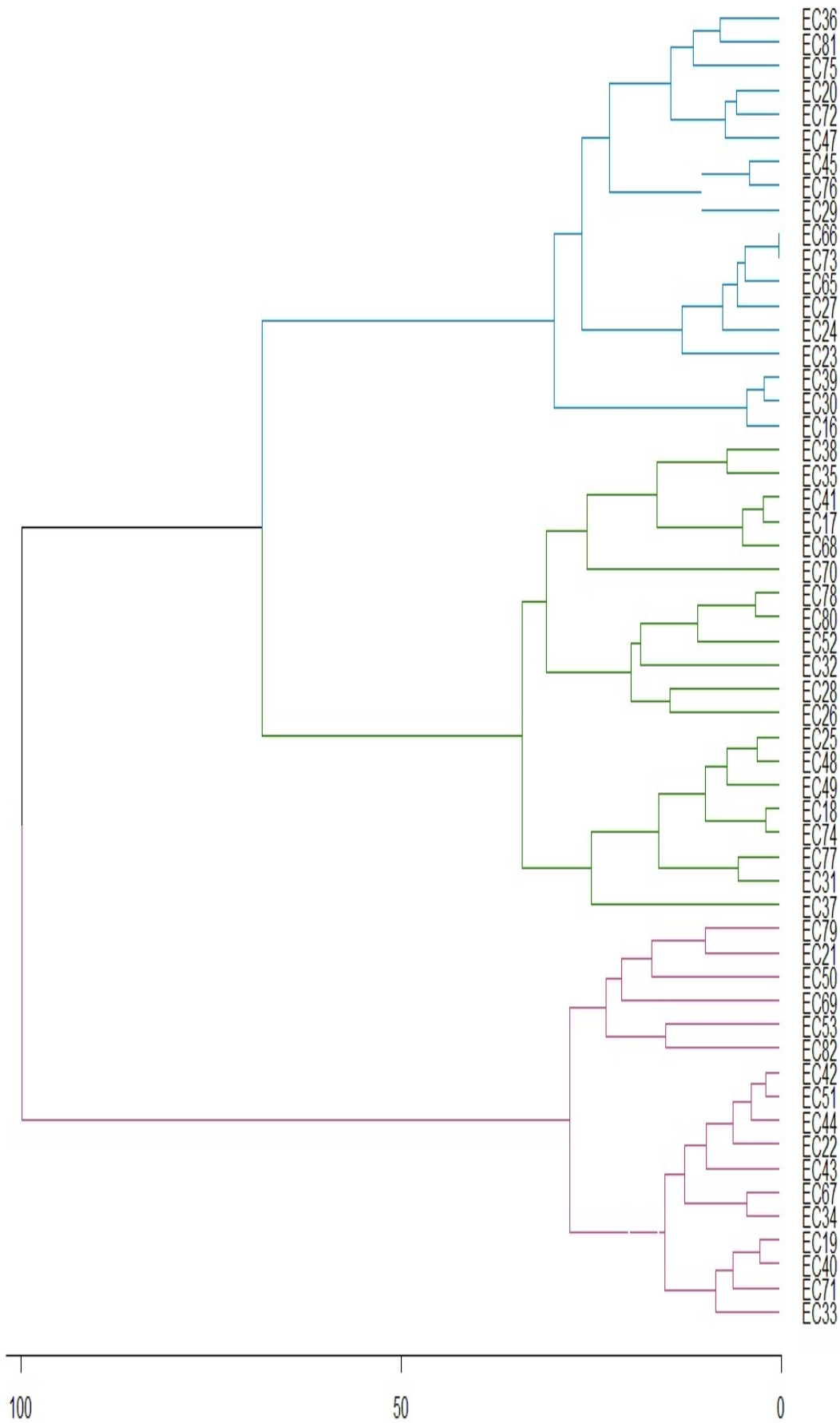
Figure 6. PCA of genotype-environment relationship of the yerba mate genotypes (EC) for total phenolic compounds concentration according to PC1 and PC2.

3.4 Proteins

Wide genetic variability was found in protein composition for yerba mate genotypes, with strong genetic influence and an increase in proteins of up to 59.49% among progenies (Table 7).

Table 7. Variance analysis associated with the breakdown of interaction effect (GxA) for proteins concentration on yerba mate genotypes (EC) tested across four consecutive years.

	High	Variance (%)		Variation sources	GL	SQ	F (Pr>F)
	Axes Value	Explained	Cumulative	Model	56	776.84	
				Genotype (G)	(53)	(354.64)	2.68 (<0.0001)
				Year (A)	(3)	(422.20)	56.31 (<0.0001)
				GxA	159	397.41	
1	195.96	49.31	49.31	IPCA1	(55)	(195.96)	3.04(<0.0001)
2	132.31	33.29	82.60	IPCA2	(53)	(132.31)	1.23 (0.1491)
3	69.14	17.40	100.00	IPCA3	(51)	(69.14)	1.06 (0.3731)
				Mean Error	290	402.90	
				Adjusted Total	215	1174.25	

GL: liberty levels; SQ: sum of the square; Pr>F: probability estimate by F-test (Fischer-Snedecor).

Principal component analysis for distribution of total protein concentrations showed a variation from 10.39 to 16.58 g 100 g^{-1} in the 54 analyzed genotypes (Table 8).

Table 8. Mean genotypic values and analysis of parameter estimates ($\mu + g$) the mean interaction GxE ($\mu + g + ge$) for total protein concentration in yerba mate genotypes (EC) tested across four consecutive years.

Genotypes	Means	Chi-Square	Pr > ChiSq	Genotypes	Means	Chi-Square	Pr > ChiSq
EC16	13.3475	0.03	0.8544	EC43	14.7075	2.78	0.0952
EC17	10.3950	8.91	0.0028	EC44	12.1425	1.05	0.3046
EC18	14.0125	0.65	0.4209	EC45	16.2075	6.97	0.0083
EC19	**11.6125**	2.54	0.1108	EC47	11.3775	3.44	0.0638
EC20	12.9750	0.24	0.6277	EC48	13.8200	0.39	0.5298
EC21	12.8350	0.10	0.7508	EC49	15.2000	3.38	0.0662
EC22	11.7525	2.08	0.1492	EC50	11.1350	4.52	0.0335
EC23	11.3125	3.71	0.0541	EC51	14.4250	1.38	0.2402
EC24	13.0875	0.55	0.4578	EC52	13.5150	0.41	0.5238
EC25	12.8925	0.01	0.9131	EC53	16.5800	8.54	0.0035
EC26	14.0875	0.76	0.3827	EC65	12.7725	0.34	0.5608
EC27	14.6025	1.77	0.1836	EC66	12.6300	0.27	0.6005
EC28	12.5550	0.34	0.5599	EC67	11.6725	1.24	0.2663
EC29	13.7550	0.74	0.3882	EC68	14.1300	0.83	0.3621
EC30	12.6575	0.47	0.4944	EC69	14.1000	0.78	0.3766
EC31	14.5175	1.58	0.2092	EC70	12.9625	0.02	0.8908
EC32	13.8325	0.41	0.5223	EC71	13.9100	0.51	0.4770
EC33	13.3525	0.04	0.8507	EC72	13.7300	0.30	0.5859
EC34	12.7325	0.05	0.8258	EC73	16.0675	4.76	0.0290
EC35	14.3075	1.15	0.2844	EC74	14.0200	0.27	0.6009
EC36	14.2100	0.97	0.3255	EC76	13.0775	0.01	0.9378

EC37	14.2125	0.97	0.3244	EC77	12.7725	0.09	0.7675
EC38	14.7925	2.23	0.1352	EC78	13.3575	0.04	0.8469
EC39	13.5625	0.15	0.6981	EC79	12.6975	0.01	0.9114
EC40	15.4900	4.31	0.0380	EC80	12.5275	0.67	0.4140
EC41	13.0575	0.01	0.9222	EC81	11.5050	2.93	0.0868
EC42	14.3575	1.24	0.2649	EC82	13.1575	0.24	0.6277

First component describes 49.31% of the variability observed in genotype-environment interaction (Figure 7).
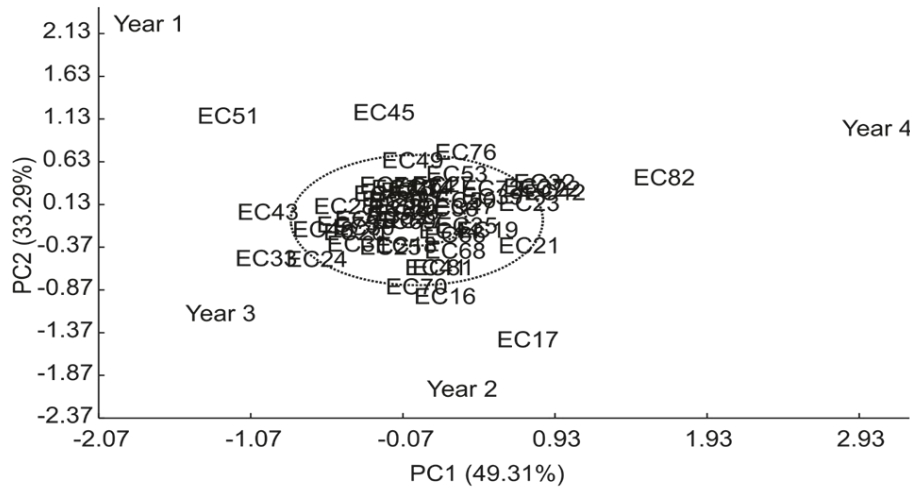
Figure 7. PCA of the first component for total protein concentration in yerba mate genotypes (EC) in 2015, 2016, 2017 and 2018.

Results show that 31.5% of genotypes were stable at 1% probability of error (represented in blue) and 42.6% were stable at 5% (represented in green), while 25.9% of genotypes were not stable (represented in red) (Figure 8).
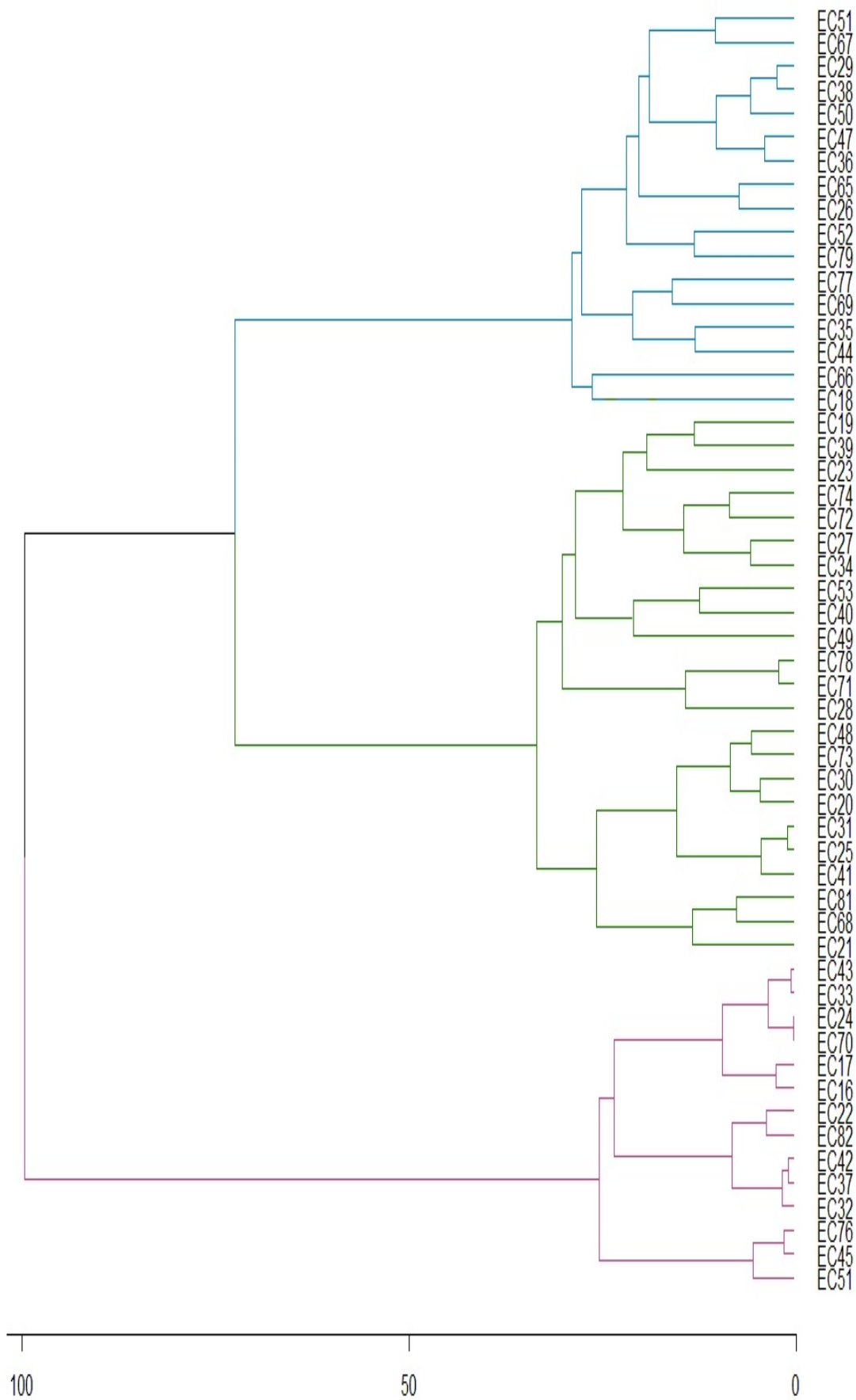
Figure 8. PCA of the genotype-environment ratio of the yerba mate genotypes (EC) for total protein concentration according to PC1 and PC2.

4. Discussion

We confirm the wide genetic variability for caffeine concentration in yerba mate. Sixteen genotypes (EC19, EC22, EC30, EC16, EC44, EC82, EC67, EC17, EC81, EC50, EC18, EC78, EC51, EC29, EC52 and EC76) presented average caffeine concentrations below 0.1 g 100 g^{-1} and, therefore can be considered decaffeinated according to Anvisa's RDC No. 277 [24]. All other 38 genotypes were characterized as caffeinated, with caffeine concentrations ranging from 0.121 to 2.385 g 100 g^{-1}. Following the classification defined for yerba mate [25], caffeine concentration can be very low or absent (< 0.1%), low (0.1 - 1.0%), medium (1.0 - 1.6%) or high (>1.6%). Such information made it possible to observe a wide distribution of the studied genotypes where four designated ranges could be found, representing 29.63% (very low), 24.07% (low), 27.78% (medium) and 18.52% (high) (Figure 1).

Caffeine is naturally present in several products and is one of the most consumed food ingredients in the world due to its presence in teas, guarana and coffee [26]. Caffeine content in teas varies greatly depending on the species, edaphoclimatic conditions of its cultivation and phenological stage of leaves [26,27]), which can influence the product quality, making it bitter and altering flavor of the final product [3,28].

Although caffeine presence in yerba mate is well known [1,7,18,29,30], the high variability among genetic materials (0.01 to 2.96 g 100 g^{-1}) are still dubious [18,31] and the lack of knowledge about its stability is clear. When these characteristics are standardized and evaluated successively over several production cycles, more reliable analyses can be established to support the emergence of new yerba mate cultivars with different caffeine concentrations, for different uses.

Over time, high complexity between samples and processing protocols has led yerba mate to be characterized as a biomass with lower levels of caffeine when compared to *Camellia sinensis*, even though it presents the highest transfer percentages during infusion (80.9 to 85.2%) [32]. Results of PCA from caffeine levels identified in this study confirm the broad potential for developing different products with low (or decaffeinated), medium and high caffeine concentrations from yerba mate biomass (Figure 1). The great dependence of genotype on caffeine concentrations verified in our study is a result of high genetic heritability (h²) for caffeine content, verified also on studies conducted on yerba mate, (h² from 0.60 to 0.83) [29,33,34].

Lower theobromine concentrations in yerba mate plants have constantly been reported [2], ranging from 0.01 to 0.95 g 100 g^{-1} [18] and 0.002 and 0.503 g 100 g^{-1} [27]. In *Camelia sinensis* plants, inversely proportional relationships between caffeine and theobromine have frequently been identified [28,35]. According to results observed herein, there was a negative correlation between both compounds; however, it was too low to draw any conclusions. Such information is based on caffeine biosynthesis, which involves a series of reactions and can culminate in the conversion of theobromine into caffeine [8,36].

The presence of polyphenols in yerba mate is well known [2,13] and has aroused interest in its antioxidant concentrations, which help protect body tissues against oxidative stress events such as cellular aging and degenerative diseases (cancer, cardiovascular diseases, inflammation, etc.) [2]. Its concentration in plant tissues is determined by several factors, including genetic [29,31] and environmental [37] variability, processing forms [38], in addition to the time between harvesting and processing plant material [27].

Given the health benefits provided by yerba mate, especially its concentration of phenolic compounds, the importance of research that can provide these compounds for human consumption is evident [39]. In extracts and commercial products, concentration of phenolic compounds is high and highly dependent on factors related to cultivation and processing [14–16], and is sometimes higher in yerba mate than in products such as green tea and red wine [40]. The presence of these compounds could be preponderant for nutritional and sensory improvement, as well as significantly increase the shelf life of industrialized products [41]. However, this characteristic fails to represent the genetic potential of yerba mate in supplying antioxidants. In our study, the amplitude of phenolic compounds distribution in yerba mate was clarified, generating new perspectives for standardized leaf biomass production with wide industrial application.

Principal component analysis for total phenolic compounds concentration agrees with the conclusion of a study conducted by Sturion and collaborators [42], that shows that total polyphenol content has low genetic control. On the other hand, the stability found for total phenolic compounds concentration in 63.64% of the evaluated genotypes over four consecutive years represents significant evolution and technical and scientific foundation in the search for clonal materials. The availability of genetic materials with high concentrations of total phenolic compounds could favor the consumption and absorption of antioxidants, without increasing the consumption volume of yerba mate products by population [43]. The known variability between products made from commercial biomass (leaves and thin branches) of yerba mate is attributed to biomass characteristics and its processing, inversely proportional to increased proportion of branches/leaves, reduction of the surface area of leaf biomass and highly sensitive to variation in the raw material production and processing [16].

Our results for total protein concentration are higher than those found for industrially processed yerba mate leaves. For example, 7.97 ± 0.17 g 100 g^{-1} from 15-year-old [44] and 10.06 ± 0.12 g 100 g^{-1} for 80-year-old [45] plants, both native from shaded environment, and 9.52 ± 0.42 g 100 g^{-1} from leaves of 12-year-old planted trees [45]. The wide genetic variability found in its concentration and its good stability over different years (around 75%) in out studied genotypes, also helps yerba mate producers do develop new cultivars with different protein concentrations, for different uses.

For the first time, this study addresses the composition of main bioactive compounds in selected *Ilex paraguariensis* genotypes and their stability over four consecutive years. The results suggest that variation in chemical composition is strongly influenced by genotype and has high stability through four successive harvests.

5. Conclusions

Our results indicate large variations between different selected *Ilex paraguariensis* genotypes regarding caffeine, theobromine, total phenolic compounds and proteins concentrations. The genetic materials are individually stable for concentration of those bioactive compounds, establishing a huge potential for innovation with the species.

Author Contributions: Study planning, selection and collection of genetic material for analysis and scientific writing, D.C.D.B, C.V.H., C.A.S. and I.W.; Methodology, C.V.H., A.C.M, R.I. and M.V.L.; Investigation, D.C.D.B, C.V.H., A.C.M, R.I. and M.V.L.; Writing – Original Draft, C.A.S.; Writing –Review & Editing, D.C.D.B, C.A.S., C.V.H., A.C.M, R.I. and M.V.L., I.W. and O.J.L.; Resources, D.C.D.B, C.V.H., C.A.S. and I.W.; Data analysis, O.J.L.; Supervision, C.V.H., I.W. and M.V.L.

Data Availability Statement: All data generated or analyzed during this study are included in this published article. The datasets generated during and/or analyzed during the current study are not publicly available due reason why data are not public, but are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval: This study is exempt from ethical approval as it does not directly or indirectly investigate human beings.

Consent for publication: This study is exempt from consent for publication as it does not directly or indirectly investigate human beings.

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