

## Article

# Diversity of Leaf Stomatal Traits among *Coffea canephora* Pierre ex A. Froehner Genotypes

Danielly Dubberstein <sup>1</sup>, Marcos Góes Oliveira <sup>2</sup> , Elisa Mitsuko Aoyama <sup>2</sup>, José Henrique Guilhen <sup>3</sup>, Adésio Ferreira <sup>3</sup>, Isabel Marques <sup>4</sup> , José C. Ramalho <sup>4,5</sup>  and Fábio Luiz Partelli <sup>2,\*</sup> 

<sup>1</sup> Instituto Federal de Rondônia and Instituição de Ensino Superior de Cacoal-Fanorte, Cacoal, Rondônia 76963-868, Brazil; dany\_dubberstein@hotmail.com

<sup>2</sup> Departamento de Ciências Agrárias e Biológicas, Centro Universitário Norte do Espírito Santo, Universidade Federal do Espírito Santo, Santo 29932-540, Brazil; mgoesoliveira@hotmail.com (M.G.O.); elisaoyama@yahoo.com.br (E.M.A.)

<sup>3</sup> Centro de Ciências Agrárias e Engenharias, Universidade Federal do Espírito Santo, Alto Universitário, Alegre 29500-000, Brazil; josehenrique@gmail.com (J.H.G.); adesioferreira@gmail.com (A.F.)

<sup>4</sup> Plant-Environment Interactions and Biodiversity Lab (PlantStress & Biodiversity), Forest Research Centre (CEF), Instituto Superior de Agronomia (ISA), Universidade de Lisboa, 1349-017 Lisboa, Portugal; isabelmarques@isa.ulisboa.pt (I.M.); cochichor@isa.ulisboa.pt (J.C.R.)

<sup>5</sup> GeoBioSciences, GeoTechnologies and GeoEngineering (GeoBioTec), Faculdade de Ciências e Tecnologia (FCT), Universidade NOVA de Lisboa (UNL), 2829-516 Monte de Caparica, Portugal

\* Correspondence: partelli@yahoo.com.br



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**Abstract:** Leaf morpho-anatomical characteristics directly reflect photosynthetic performance and the ability to adapt to different environmental conditions. The study of biometric traits is essential for the selection of promising plant materials for breeding purposes. To identify new varieties of coffee plants with desirable traits for genetic improvement programs, this study investigated the variability of leaf morpho-anatomical traits in 43 genotypes of *Coffea canephora* (as the species under study is hypostomatous). Seven leaf characteristics were used: epidermal cell density (ECD), stomatal length (SL), stomatal width (SW), stomatal density (SD), stomatal size (SS), stomatal index (SI), and stomatal length/width. Morphological traits (plant height, internodal distance, and leaf area) and grain production were also assessed. The data analyzed multivariate analysis of variance grouped by the unweighted pair group the arithmetic mean hierarchical method, and data were also subjected to a Pearson linear correlation and principal component analyses (PCAs). The results showed wide morphological variability reflecting six morphological groups, which is relevant for the genetic divergence analysis and for breeding purposes, as the results have the potential to identify superior genotypes. Within the groups, genotypes were mainly separated by the number of epidermal cells and the number and size of the stomata, reflecting a high genetic heterogeneity within genotypes. Positive and negative correlations were found, with levels of significance ranging from weak to strong among the analyzed traits. The highest correlation levels were found for SL × SS, SW × SS, and SI × SD. In addition, the PCA indicated that plant height, distance between nodes, and leaf area were positively correlated and associated. The greater the number and width of stomata, the higher the rate of gas exchange. Both characteristics are favorable for the development and production of coffee plants, explaining the positive correlation observed in this study. These results emphasize the usefulness of trait evaluations for the identification and breeding of genotypes to compose new *C. canephora* cultivars suitable for changing environments.

**Keywords:** biodiversity; biometrics; clustering; multivariate analysis; stomata; variability

## 1. Introduction

Leaf characteristics are among the most important groups of plant functional traits and are a robust proxy for the features of the entire plant [1,2]. Leaf traits have been extensively analyzed to discern how plant species coexist within a community and adapt to changing

environments [3] and to understand genotype variability in crop breeding lines [4]. As temperature and drought events continue to increase in number and intensity, it becomes critical to develop crops with improved water-use efficiency (WUE) under the predicted future climatic conditions [5]. Leaf morpho-anatomical characteristics directly reflect photosynthetic performance and the ability to adapt to different environmental conditions. Studies show that changes in leaf thickness and increased chlorophyll content, for example, contribute to the enhancement of the light capture and, as a result, to photosynthesis [6].

Coffee is one of the most valuable agricultural products in the global economy, being cultivated over an area exceeding 11 million hectares and distributed across ca. 80 countries in the tropical region [7,8], with the species *Coffea arabica* L. and *C. canephora* Pierre ex A. Froehner dominating the global coffee cultivation and trade. The production and the demand of *C. canephora* have significantly increased due to its use in blends with *C. arabica* (both for espresso and soluble coffees) because of its higher content of soluble solids, greater industrial yield, and its contribution to a full-bodied beverage [9]. Some authors argue that this can provide up to 60% of the blend percentage without compromising the final beverage quality [10].

*Coffea canephora* is a diploid species ( $2n = 2x = 22$  chromosomes), mainly allogamous, with a monogenic gametophytic self-incompatibility linked to a single “S” gene locus, where at least three alleles interact. Therefore, neither self-fertilization nor crossing of individuals with similar alleles occurs [7,11,12]. It was found that natural cross-breeding promotes highly heterozygous individuals and populations with a high genetic variability [13–15] in plant features such as resistance to environmental biotic and abiotic stresses, overall architecture, cycles of maturation, productivity, size and shape of seeds, fruits, and leaves, and even the quality of the beverage [13,16,17]. *Coffea canephora* genotypes (mostly from Conilon and Robusta cultivars) are frequently selected in the field, mostly through farmers’ empirical selection and multiplication of plant material. Some tolerance characteristics to harsh environmental conditions are observed in the field, such as those related to low or high temperatures or drought, according to genotype [18]. Plants with such contrasting traits are key in breeding programs because they can be used to develop elite varieties of coffee, allowing for combining a wide genetic diversity with the high performance of plants that are better adapted to changing environmental conditions [13,18,19]. The evaluation of agronomic characteristics using multivariate techniques can identify promising and/or distinct genotypes to develop new stable cultivars with homogeneity in their development, as well as better coffee bean quality and higher crop yield.

Stomata are structures found in the plant epidermis, mainly in the leaves. These specialized structures ensure that gas exchange between the plant and the environment occurs. In this process, carbon dioxide enters, driving plant growth and transpiration, which regulates nutrient absorption, leaf temperature, and root-to-root signaling. The stomatal density and size of the stomata can either positively or negatively influence gas exchange [19].

Previous studies have shown that stomatal features can effectively explain the disparity between coffee genotypes [20]. Therefore, this study aims to evaluate the diversity of morphological leaf traits, particularly those related to the stomata, of 43 genotypes of *C. canephora* cv. Conilon, grown in a typical cropping region in Brazil. Leaf characteristics can vary between coffee genotypes and are useful in identifying plants with desirable traits, such as resistance to some environmental conditions. In fact, the leaf anatomy of coffee plants shows interesting plasticity under exposure to abiotic stresses through the alteration of the thickness of the palisade and spongy parenchyma and of the number, positioning, and size of stomata [21]. These characteristics can aid in the selection of genotypes with greater photosynthetic potential, such as those having an increased stomatal density that can improve CO<sub>2</sub> availability to the carboxylation sites and, therefore, increase photoassimilation [22,23].

## 2. Material and Methods

### 2.1. Plant Material and Field Characterization

This study was performed in a cropped area with 43 genotypes of *C. canephora* Pierre ex A. Froehner cv. Conilon, most of which were selected by coffee growers in the region due to their yield and quality performance (Table 1). Planting occurred in April 2014 in Nova Venécia, in the northern region of the State of Espírito Santo, Brazil (18°39'43" S, 40°25'52" W; 199 m a.s.l.) with a mean annual temperature of 23 °C. The region has a tropical climate, characterized by hot and humid summers and dry winters (classified as Aw, according to the Köppen classification [24] with an average annual precipitation of 1200 mm, although the entire area benefits from sprinkler irrigation. The soil is Latossolo Vermelho-Amarelo, dystrophic, with a clayey texture and wavy relief [25].

**Table 1.** Identification of the 43 genotypes of *Coffea canephora* cv. Conilon in Nova Venécia, ES, Brazil.

Identification	Name	Identification	Name	Identification	Name
1	Verdim R	16	Pirata	31	Cheique
2	B01	17	Peneirão	32	P2
3	Bicudo	18	Z39	33	Emcapa 02
4	Alecrim	19	Z35	34	Emcapa 153
5	700	20	Z40	35	P1
6	CH1	21	Z29	36	LB1
7	Imbigudinho	22	Z38	37	122
8	AD1	23	Z18	38	Verdim D
9	Graudão HP	24	Z37	39	Seed
10	Valcir P	25	Z21	40	Emcapa 143
11	Beira Rio 8	26	Z36	41	Ouro Negro 1
12	Tardio V	27	Ouro Negro	42	Ouro Negro 2
13	AP	28	18	43	Clementino <sup>T</sup>
14	L80	29	Tardio C	-	-
15	Bamburral	30	A1	-	-

Genotype 33 belongs to cv. Emcapa 8111 and genotypes 34 and 39 to cv. Emcapa 8131 [13]. Genotypes 1, 11, 15, 16, 30, and 42 belong to cv. Tributun [20,26], and 30 and 35 to cv. Andina [27–29].

The genotypes were arranged in a randomized block design with three replications, each with seven plants of each genotype. Planting was carried out at a spacing of 3 m between lines and 1 m within the line, resulting in a density of 3333 plants per hectare. All genotypes were propagated by cuttings, except for genotype 39, which was propagated by seeds. Plants were managed to have four stems per plant. The entire experimental area was irrigated using a drip irrigation system.

The plants received 500, 100, and 400 kg ha<sup>-1</sup> year<sup>-1</sup> of N, P<sub>2</sub>O<sub>5</sub>, and K<sub>2</sub>O, respectively, depending on the plant requirements and phenological stages; there were also amounts of 2 kg ha<sup>-1</sup> year<sup>-1</sup> Zn, 1.0 kg ha<sup>-1</sup> year<sup>-1</sup> B, 2.0 kg ha<sup>-1</sup> year<sup>-1</sup> Cu, and 10 kg ha<sup>-1</sup> year<sup>-1</sup> Mn.

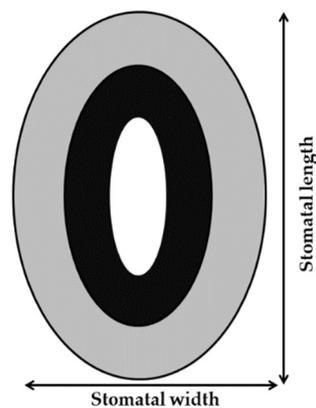
### 2.2. Leaf Morphological and Anatomical Traits Determination

Newly developed leaves from the third and fourth pairs of plagiotropic branches were collected from the middle third of the plants, with a total of 20 leaves per genotype on 1 February 2017. The central part of each leaf was immediately cut and fixed in FAA 50 solution (formaldehyde, glacial acetic acid, and 50% ethanol, 1:1:9, v/v) for 48 h and preserved in 70% ethanol thereafter. Leaf samples of approximately 1 cm<sup>2</sup> were sequentially treated, first in a 10% sodium hydroxide solution, followed by a 50% sodium hypochlorite solution, washed in distilled water, stained with 1% safranin, and placed on glass slides with water [29].

Leaf area was assessed from another set of 20 leaves per genotype using a leaf area meter (Model LI-3100, Li-Cor, Lincoln, NE, USA). Plant biometric analyses included plant height (measured from the base to apex of the canopy in cm), the number of nodes in

the plagiotropic productive branches, and the mean internode length of these branches (by dividing the branch length by the number of nodes). These measurements were in six plants per genotype. The crop yield was assessed annually for four years (2015, 2016, 2017, and 2018), and the average crop yield within these years was used to calculate the correlations with shoot traits. Three plants in each experimental unit were assessed in three replicates, totaling nine plants for each genotype [30].

A total of five microscope slides per genotype were observed and photographed using an optical microscope (Motic BA210, equipped with a Motic Cam 3<sup>®</sup> 3.0 MP camera and Motic Images Plus 2.0 software). From each slide, photographic images of 10 distinct areas of the abaxial leaf side were taken for each genotype. After that, 50 photographs per genotype were analyzed with the Anati Quanti program [31] to quantify the epidermal cell density (ECD; in cells mm<sup>-2</sup>), stomatal length (SL; at the major axis) and width (SW; at minor axis) in  $\mu\text{m}$  (both in Figure 1), and the density (SD); these reflect the number of stomata per unit of leaf area, as well as the stomatal size (stomatal length  $\times$  stomatal width (SS), stomatal index (SI, calculated as the ratio between stomatal number (NS) and the sum of epidermal cells with NS), and stomatal length/width (SLW; indicative of shape) [32–35]. The parameters were estimated according to Sack and Buckley [35].



**Figure 1.** Schematic representation of the stomatal dimensions as measured by image analysis. The grey area represents the guard cells, the black area the pore walls, and the white area (internal ellipse) depicts the stomatal pore area.

### 2.3. Statistical Data Analysis

The average of all leaf traits was calculated at the genotype level. The following variance components were also estimated: coefficient of environmental variation ( $CV_e$ ), coefficient of genetic variation ( $CV_g$ ), variation index (VI, corresponding to the  $CV_g$  and  $CV_e$  ratio), and heritability ( $h^2$ ). We then compared the differences among genotypes using an ANOVA test followed by the Scott–Knott test at a 1% probability.

We performed a multicollinearity test with the following variables: ECD, SD, SL, and SW. As the NC value was less than 100, which is considered a weak degree of multicollinearity, we continued the study [32]. The generalized Mahalanobis distance matrix (D2) was used as a dissimilarity measure, and genotype clustering was performed using both the Tocher optimization method and the unweighted pair group with arithmetic mean hierarchical clustering method (UPGMA). Subsequently, the variables were subjected to Pearson's correlation analysis. A principal component analysis (PCA) in a dispersion plot of the Biplot type was also performed. Statistical analyses were performed using the R program [36].

## 3. Results

### 3.1. Morpho-Anatomical Characterization

When analyzing the images generated by the Anati Quanti program we found that the stomata were present only in the abaxial of the coffee leaves. Significant differences were

detected among the 43 genotypes of *C. canephora* based on an F-test at 1% and considering all the measured leaf traits (Table 1). The coefficient of environmental variation ( $CV_e$ ) ranged from 4.83% in SL to 14.5% in SD. The SD variable showed a value >10%, while the other traits were concentrated in low experimental  $CV_e$  values < 10% (Table 2).

**Table 2.** Summary of analysis of variance for the leaf morpho-anatomical traits evaluated in 43 genotypes of *Coffea canephora*.

Variables	MS		Mean	$CV_e$ (%)	$CV_g$ (%)	VI	$h^2$ (%)
	Genotype	Residual					
ECD	116,920 **	82.2	102.0	8.84	8.29	0.93	93.0
SI	47.1 **	4.80	23.2	9.42	7.22	0.76	89.8
SD	21,576 **	1670	282.2	14.50	12.9	0.89	92.3
SS	21,322 **	1337	435.0	8.40	8.38	0.99	93.7
SL	24.5 **	1.55	25.7	4.83	4.80	0.99	93.7
SW	8.03 **	0.768	16.9	5.19	4.12	0.79	90.4
SLW	0.390 **	0.007	1.52	5.68	3.03	0.53	81.1

\*\* Significant at 1% by F-test;  $CV_e$ , environmental coefficient of variation;  $CV_g$ , genetic coefficient of variation; VI, variation index ( $CV_g/CV_e$ );  $h^2$ , heritability; ECD, epidermal cell density (cells  $mm^{-2}$ ); SI, stomatal index (%); SD, stomatal density (unit  $mm^{-2}$ ); SS, stomatal size (stomatal length  $\times$  stomatal width); SL, stomatal length (major axis, in  $\mu m$ ); SW, stomatal width (minor axis, in  $\mu m$ ); SLW, stomatal length/width (indicative of shape).

The coefficient of genetic variation ( $CV_g$ ), quantifying the influence of the genetic components for each trait, ranged from 3.03% in SLW to 12.9% in SD, but it remained below 10% for most traits (Table 1). The VI values ( $CV_g/CV_e$ ) varied between 0.53 in SLW to 0.99 in SS and SL (SLW), whereas heritability ( $h^2$ ) ranged between 81.1% in SLW and 93.7% in SS and SL, with six of the evaluated traits showing values above 90%.

Leaf morpho-anatomical traits varied significantly among the 43 analyzed genotypes (Table 3). The Scott–Knott tests allowed the detection of variability between genotypes for all leaf morpho-anatomical traits, assembling the genotypes in up to six groups (Table 3).

**Table 3.** Mean values for the leaf morpho-anatomical traits evaluated in 43 genotypes of *Coffea canephora*. The different letters indicate significant differences the genotypes according to the Scott–Knott test.

Genotype	ECD	SL	SW	SD	SS	SI	SLW
1	112.4 b	24.8 d	16.3 c	336.7 a	407.2 d	24.7 b	1.52 c
2	118.2 a	23.8 e	16.0 d	288.9 c	382.7 e	21.0 d	1.48 d
3	100.0 c	23.6 e	15.7 d	253.5 d	372.0 e	21.6 d	1.51 d
4	108.6 b	23.7 e	15.7 d	295.6 c	375.2 e	22.9 c	1.52 c
5	110.0 b	24.4 d	16.1 d	262.9 c	396.3 e	20.0 d	1.51 d
6	121.6 a	24.5 d	16.4 c	344.5 a	405.1 d	23.6 b	1.49 d
7	102.0 c	25.2 d	17.1 b	266.9 c	433.4 c	22.3 c	1.47 d
8	106.3 b	25.3 d	16.6 c	320.7 b	421.2 d	24.7 b	1.53 c
9	98.4 c	26.0 c	16.8 c	244.9 d	439.3 c	21.3 d	1.55 c
10	87.8 e	28.4 a	16.6 c	254.7 d	475.4 b	23.9 b	1.71 a
11	102.7 c	26.1 c	16.0 d	304.8 b	421.1 d	24.5 b	1.63 b
12	101.1 c	25.1 d	16.7 c	276.2 c	420.1 d	22.9 c	1.51 d
13	92.9 d	27.8 a	18.2 a	227.8 d	507.9 a	21.1 d	1.53 c
14	96.4 d	28.0 a	18.2 a	342.7 a	512.0 a	27.9 a	1.54 c
15	99.0 c	26.0 c	16.7 c	268.9 c	435.4 c	23.9 b	1.56 c
16	102.3 c	25.1 d	16.8 c	285.2 c	424.6 d	23.3 c	1.49 d
17	94.3 d	27.0 b	18.1 a	241.3 d	491.5 b	21.8 d	1.49 d
18	98.9 c	25.3 d	16.8 c	267.1 c	429.0 d	24.0 b	1.51 d
19	123.0 a	23.5 e	15.4 d	358.6 a	364.3 e	24.1 b	1.52 c
20	108.9 b	24.9 d	15.8 d	270.6 c	394.1 e	21.3 d	1.58 b
21	110.8 b	25.6 d	17.0 b	360.4 a	436.1 c	26.2 a	1.51 d
22	113.2 b	25.9 c	17.6 b	269.4 c	458.4 c	20.7 d	1.47 d
23	95.4 d	26.9 b	17.2 b	252.9 d	464.2 c	22.5 c	1.56 c

Table 3. Cont.

Genotype	ECD	SL	SW	SD	SS	SI	SLW
24	97.5 d	28.0 a	17.2 b	293.8 c	484.0 b	24.7 b	1.63 b
25	122.0 a	26.1 c	17.3 b	341.4 a	454.5 c	23.4 c	1.51 d
26	111.4 b	25.8 c	17.4 b	357.3 a	451.9 c	25.9 a	1.49 d
27	105.0 c	27.0 b	18.2 a	281.6 c	493.9 b	22.7 c	1.48 d
28	100.3 c	26.2 c	18.2 a	262.6 c	480.0 b	22.3 c	1.44 d
29	104.0 c	25.7 c	17.2 b	251.1 d	442.5 c	20.7 d	1.50 d
30	105.3 c	25.5 d	16.5 c	315.2 b	424.3 d	24.7 b	1.55 c
31	93.4 d	25.3 d	16.4 c	249.8 d	418.4 d	22.5 c	1.54 c
32	93.8 d	26.5 c	17.0 b	270.6 c	454.0 c	23.9 b	1.56 c
33	91.2 d	26.8 b	17.7 a	220.8 d	476.2 b	23.4 c	1.52 c
34	102.6 c	22.7 f	16.0 d	268.1 c	363.9 e	22.1 c	1.42 d
35	104.0 c	26.0 c	16.7 c	272.0 c	435.6 c	23.3 c	1.56 c
36	104.1 c	26.2 c	16.8 c	251.8 d	442.4 c	22.0 c	1.56 c
37	84.2 e	27.4 b	17.0 b	233.3 d	466.5 c	23.1 c	1.62 b
38	97.7 d	25.9 c	17.1 b	299.9 b	444.0 c	25.1 b	1.52 c
39	96.7 d	25.9 c	17.5 b	266.9 c	453.8 c	23.1 c	1.48 d
40	96.5 d	24.9 d	16.6 c	326.8 b	417.4 d	27.1 a	1.50 d
41	102.0 c	25.1 d	16.3 c	239.4 d	413.0 d	20.5 d	1.54 c
42	95.7 d	24.1 e	15.7 d	292.0 c	381.6 e	25.0 b	1.53 c
43	97.6 d	26.1 c	16.9 b	246.8 d	444.2 c	21.6 d	1.54 c

Mean values followed by the same letter in the column belong to the same group by the Scott–Knott test at 1% probability. ECD, epidermal cell density (cells mm<sup>-2</sup>); SI, stomatal index (%); SD, stomatal density (unit mm<sup>-2</sup>); SS, stomatal size (stomatal length × stomatal width); SL, stomatal length (major axis, in μm); SW, stomatal width (minor axis, in μm); SLW, stomatal length/width (indicative of shape).

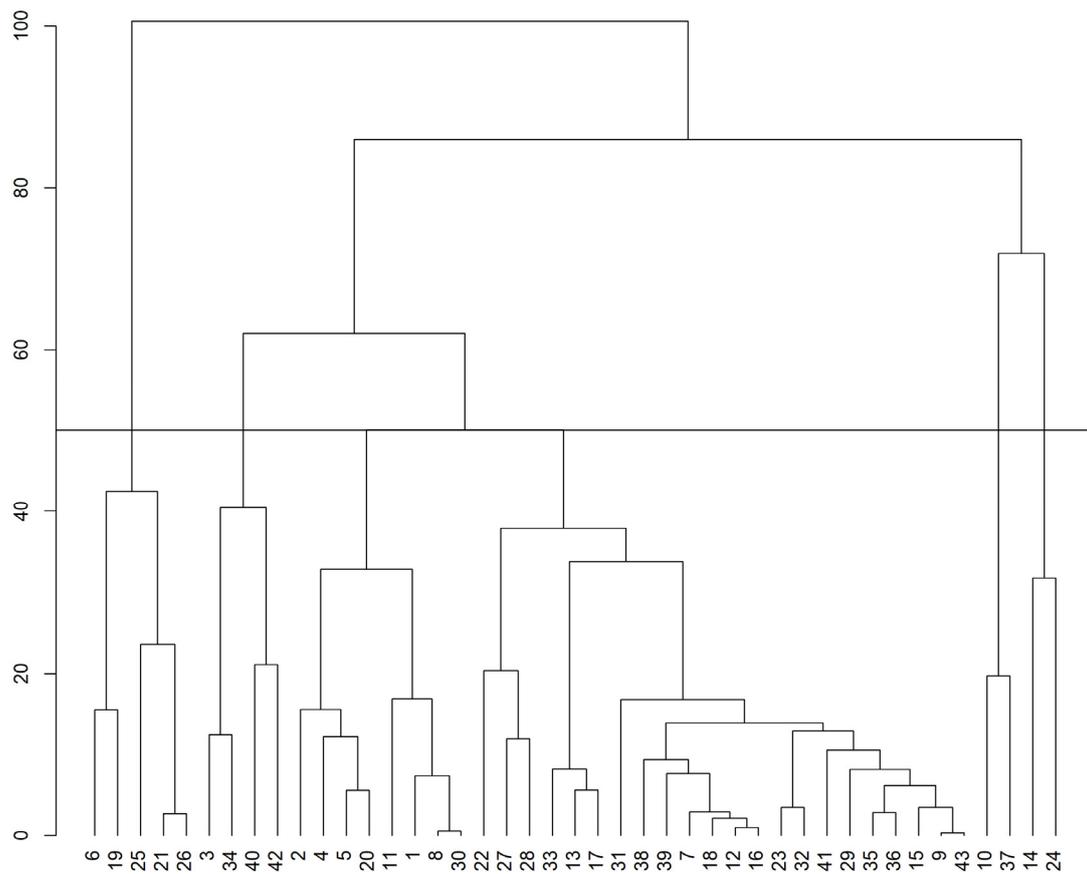
The ECD divided the 43 genotypes into five groups, ranging from 84.2 (genotype 37) to 123.0 (genotype 19). For SL, six distinct groups were formed, with genotypes 10, 13, 14, and 24 with diameters between 28.4 and 27.8 μm, and 34 (22.7 μm) showed the lowest value. For SW, the variation ranged from 18.2 μm (genotype 28) to 15.4 μm (genotype 19), clustering the genotypes into four distinct groups. The SD ranged from 360.4 (genotype 21) to 220.8 (genotype 33) stomata mm<sup>-2</sup>, with the formation of four different groups. Similarly, SI ranged from 27.9% to 20.0% (genotypes 14 and 5, respectively). The lowest observed value of SLW was 1.42, which was found in genotype 34, a fact attributed to the smaller SL because higher values of the SL/SW ratio indicate a greater functionality of the stomata by its ellipsoid shape.

### 3.2. Dissimilarity among Genotypes

The clustering analysis based on the UPGMA method and using four leaf morpho-anatomical traits revealed six distinct groups considering a 50% threshold (Figure 2). The mean value of each UPGMA cluster was calculated for these traits: ECD, SD, SL, and SW (Table 4).

**Table 4.** Means of the leaf morpho-anatomical traits of *Coffea canephora* per UPGMA cluster considering ECD (epidermal cell density; cells mm<sup>-2</sup>), SD (stomata density; unit mm<sup>-2</sup>), SL (stomatal length; at major axis in μm), and SW (stomatal width; at minor axis in μm).

Groups	Genotypes	ECD	SD	SL	SW
1	6, 19, 25, 21, 26	118.0	352.4	25.1	16.7
2	3, 34, 40, 42	98.7	285.1	23.8	16.0
3	1, 2, 4, 5, 8, 11, 20, 30	109.6	299.4	24.8	16.1
4	7, 9, 12, 13, 15, 16, 17, 18, 22, 23, 27, 28, 29, 31, 32, 33, 35, 36, 38, 39, 41, 43	99.5	259.6	26.1	17.2
5	10, 37	86.0	244.0	27.9	16.8
6	14, 24	97.0	318.2	28.0	17.8



**Figure 2.** Dendrogram representative of genetic dissimilarity among 43 genotypes of *Coffea canephora*, obtained by the UPGMA clustering method, using the generalized distance of Mahalanobis ( $D^2$ ) and a cut-off point at 50%, considering four leaf morpho-anatomical traits, i.e., ECD, epidermal cell density (cells  $\text{mm}^{-2}$ ); SD, stomata density (unit  $\text{mm}^{-2}$ ); SL, stomatal length (major axis;  $\mu\text{m}$ ); SW, stomatal width (minor axis;  $\mu\text{m}$ ).

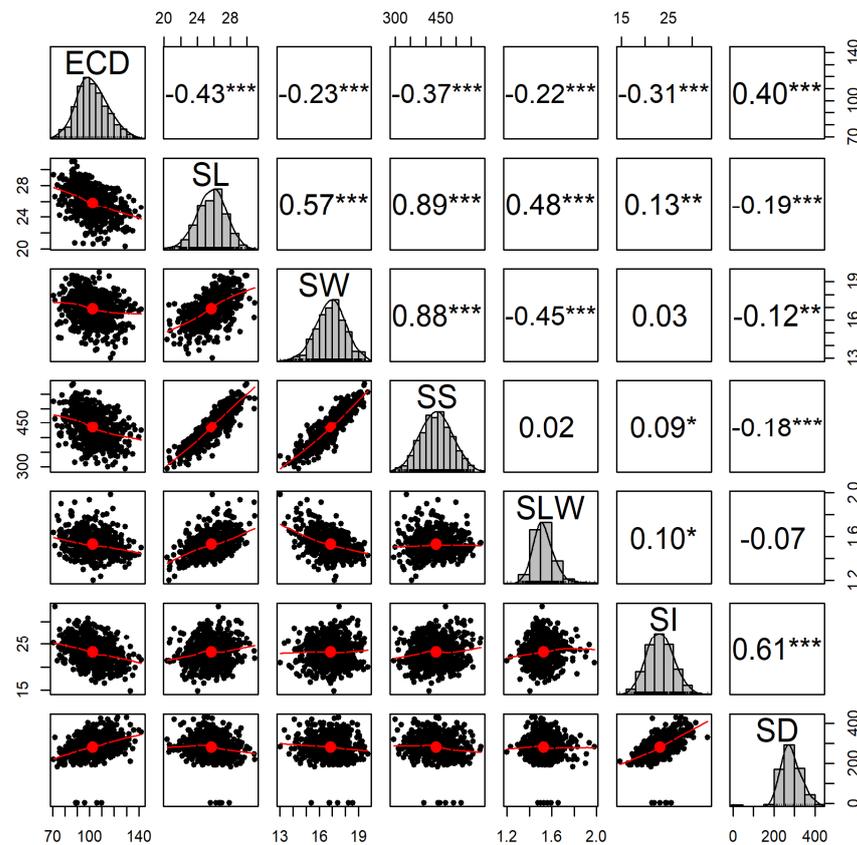
The first group, which included genotypes 6, 19, 25, 21, and 26, had the greatest ECD and SD values. The second group characterized four genotypes (3, 34, 40, and 42), which had the lowest values of SL and SW. The third group contained eight genotypes (1, 2, 4, 5, 8, 11, 20, and 30), with higher ECD (the second highest) and smaller stomata (the lowest SL and SW). The fourth group comprised approximately half of the studied genotypes, totaling 21 genotypes (7, 9, 12, 13, 15, 16, 17, 18, 22, 23, 27, 28, 29, 31, 32, 33, 35, 36, 38, 39, 41, and 43).

The fifth group was formed only by genotypes 10 and 37, characterized by lower ECD and SD, and the sixth group contained only genotypes 14 and 24, which had the highest SL and SW and the third largest SD.

### 3.3. Correlation Studies of Biometric Variables

The correlation analysis of ECD, SL, SW, SD, SS, SI, and SLW resulted in 17 significant positive and negative correlations (Figure 3). Correlation values were low, but some were significant: SL  $\times$  SI (0.13) with  $p < 0.01$ ; SL  $\times$  SD ( $-0.19$ ) with  $p < 0.001$ ; SW  $\times$  SD ( $-0.12$ ) with  $p < 0.01$ ; SS  $\times$  SI (0.09) with  $p < 0.05$ ; SS  $\times$  SD ( $-0.18$ ) with  $p < 0.001$ ; and SLW  $\times$  SI (0.10) with  $p < 0.05$ . Negative correlations were found for ECD, SW, SS, SL, SW, SD, and SS and SD. Positive correlations indicated that an SI increase was accompanied by increases in SL and SS.

There were also significant positive correlations between stomatal length traits and crop yield (0.31) with  $p < 0.05$ , as well as between stomatal index and crop yield (0.34) with  $p < 0.05$  (Table 5).



**Figure 3.** Correlations between the leaf morpho-anatomical traits: ECD, epidermal cell density (cells mm<sup>-2</sup>); SI, stomatal index (%); SD, stomatal density (unit mm<sup>-2</sup>); SS, stomatal size (stomatal length × stomatal width); SL, stomatal length (major axis; μm); SW, stomatal width (minor axis; μm); SLW, stomatal length/width (indicative of shape) of 43 *Coffea canephora* genotypes. (\*, \*\*, and \*\*\* correspond to significance of  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ , respectively).

**Table 5.** Correlations between the yield and leaf morpho-anatomical traits of 43 *Coffea canephora* genotypes.

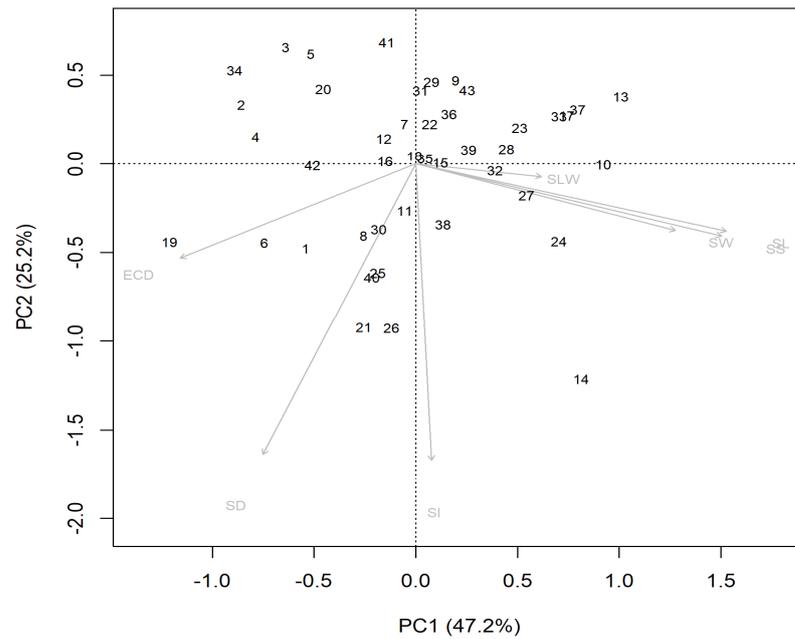
Yield	Morpho-Anatomical Traits						
	ECD	SL	SW	SS	SI	SD	SLW
	-0.13	0.31 *	0.16	0.25	0.34 *	0.13	0.24

\* Significant positive correlations with  $p < 0.05$ ; ECD, epidermal cell density (cells mm<sup>-2</sup>); SI, stomatal index (%); SD, stomatal density (unit mm<sup>-2</sup>); SS, stomatal size (stomatal length × stomatal width); SL, stomatal length (major axis; μm); SW, stomatal width (minor axis; μm); SLW, stomatal length/width.

A principal component analysis (PCA) using the same eight leaf morpho-anatomical traits (Figure 4) showed that the first two components (PC1 and PC2) explained 72.4% of the total variation. The wide genotype dispersion across the plot indicated considerable divergence in the studied biometric variables. In the biplot graph of the PCA, the variables are represented by vectors and genotypes by numbers. The larger the vector, the greater the influence of the variable in the cluster, and the smaller the angle between the vectors, the greater the correlation among variables.

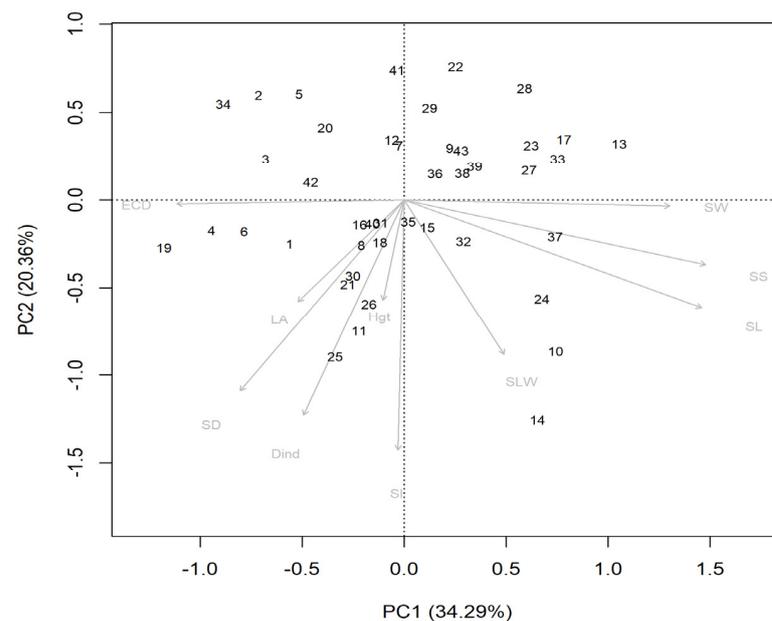
In PC1, genotypes 13 and 19 had the greater distances. This was likely related to smaller polar (23.5 μm) and equatorial (15.4 μm) diameters observed in genotype 19, which contrasted with the larger polar (27.8 μm) and equatorial (18.2 μm) diameters of genotype 13.

In PC2, a greater distance occurred between genotypes 3 and 14. This large difference relates to the lower polar diameter (23.66 μm) found in genotype 3, while genotype 14 presented the larger polar (28 μm) and equatorial (18.25 μm) diameters.



**Figure 4.** Principal component analysis considering leaf morpho-anatomical traits: ECD, epidermal cell density (cells  $\text{mm}^{-2}$ ); SI, stomatal index (%); SD, stomatal density (unit  $\text{mm}^{-2}$ ); SS, stomatal size (stomatal length  $\times$  stomatal width); SL, stomatal length (major axis;  $\mu\text{m}$ ); SW, stomatal width (minor axis;  $\mu\text{m}$ ); SLW, stomatal length/width of 43 *Coffea canephora* genotypes.

An additional PCA analysis was performed by adding plant height (Hgt), distance internodes (Dind), and leaf area (LA) to the previous eight foliar anatomical variables in order to reveal possible relationships between these plant traits (Figure 5). The two components (PC1 and PC2) of the PCA explained 54.6% of the total variation, with the anatomical variables in the PC1 being negative and PC2 being negative quadrants along with SD and SI. The leaf area was close to the number of epidermal cells, while the intermodal distance was maintained along with SD, and plant height was between SI and SD.



**Figure 5.** Principal components analysis considering eight leaf anatomical variables and three biometric characteristics of 43 *Coffea canephora* genotypes.

## 4. Discussion

### 4.1. Morpho-Anatomical Characterization

Our data confirmed that coffee is a hypostomatic species [29]. The presence of stomata of coffee leaves is crucial as it affects fundamental biological processes. For example, in maize leaves, photosynthesis and transpiration rates are higher on the abaxial surface, showing a stable or even increasing uptake of CO<sub>2</sub> as the concentration increased, as compared to the adaxial surface where CO<sub>2</sub> uptake values were progressively inhibited at concentrations above the growth CO<sub>2</sub> value [26,29].

Significant differences were found among the 43 genotypes of *C. canephora* for all leaf morpho-anatomical traits studied (Table 2), using the F-test at 1% probability. This highlights the presence of morphological diversity among the studied genotypes, which could be used in breeding programs where the existence of genetic variability is essential [19,37–39]. Variation in morphological traits was also previously found among three cultivars of *C. arabica*, considering the stomatal density (SD) [40]. The morphological traits of stomata, such as size, shape, frequency, and distribution, are considered species dependent, but they can greatly vary among genotypes under the same environmental conditions, or within the same genotype when subjected to different environmental factors, with the latter demonstrating the ability of plants to adjust to external pressures, namely increased temperature [21], lower water availability [26,41], elevated atmospheric CO<sub>2</sub> [37,42], high relative air humidity [43,44], and differing irradiance levels [40]. Environmental changes can alter stomatal development and patterning in the epidermis of new leaves [45,46]. For example, exposure of leaves to high CO<sub>2</sub> reduced stomatal density (SD) and stomatal index (SI) in poplar [47]. Furthermore, in the present study, SD was also observed to decrease while stomatal size increased in coffee, although there was variation between genotypes, whereas no changes were found in *C. canephora* cv. Conilon Clone 153 for these stomatal parameters under long-term elevated CO<sub>2</sub> conditions [29]. In addition, stomata play a fundamental role in regulating plant water use and carbon gain, constituting a key target for improving WUE [48].

The coefficient of variation (CV%) is an estimate of the experimental error in relation to the overall mean of the trial. The lower the CV%, the greater the precision of the experiment, indicating improved experimental quality and smaller differences between significant mean estimates, and vice versa [49]. In this context, the values obtained for the evaluated traits were within acceptable levels in the field experiment, indicating good accuracy and experimental precision [14,50]. In fact, the coefficient of variation parameters, CV<sub>e</sub> (reflecting how much the environment influences the evaluated characteristics expression) and CV<sub>g</sub> (quantifying the genetic component influence for each trait), showed values in the ranges of 4.83% (SL) to 14.5% (SD), and of 3.03% (SLW) to 12.9% (SD), respectively. These values can be considered low (below 10%) for most traits, with the exception of stomatal number (NS) and SD, which is considered medium (between 10% and 20%) by some authors [34], while CV values above 7% are considered high by other authors [51,52]. This was the case in five out of the eight leaf morpho-anatomical traits evaluated [26]. The CV<sub>g</sub> allows us to infer the magnitude of the variability present in the population, making it possible to compare the genetic variability levels of different genotypes, environments, and traits [14]. The higher the CV<sub>g</sub> value, the more heterogeneous the genotypes evaluated [53,54].

The VI values (CV<sub>g</sub>/CV<sub>e</sub>) ranged from 0.53 in SLW to 0.99 in SS and SL, where most traits values were close to 1, which is important for the selection of superior genotypes. In fact, values within the range of 0.70–2.00 are considered useful for breeding programs [14], with those similar to or above 1.0 revealing genotypes favorable for genetic improvement [55], since variation of a specific trait is greater than environmental variation. Additionally, six of the eight evaluated traits heritability values (h<sup>2</sup>) above 90%. These high values suggest a strong possibility of genetic gains in the selection procedures [53], a high additive genetic variance, lower environmental variation, and less environmental genotype interaction [56].

#### 4.2. Dissimilarity between Genotypes

The UPGMA hierarchical method based on the generalized distance of Mahalanobis (D2) followed the criterion that the average of the measures of divergence within each group should be smaller than the average distances between any groups [57]. To perform this procedure, we used only the variables ECD, SL, and SW (Figure 2), discarding the highly correlated variables in order to avoid redundancy, as they are not necessary in studies of genetic divergence [58]. Only the characteristics representing the fundamental structure of the biological system under study should be retained in the analysis, provided they are sufficiently diverse to represent at a minimum the most important dimensions. In this context, the clustering analysis using the UPGMA method allowed for the formation of six distinct groups regarding the leaf morpho-anatomical traits while considering a 50% cut-off, as recommended by Mojena (1977) [59], as seen in Figure 2. This is not a high number of groups considering the number (43) of analyzed genotypes. For instance, seven distinct groups were obtained using the UPGMA method in only 12 accessions of *C. arabica* L. var. Maragogipe Hoert. ex Frohner, and using 27 morpho-agronomic descriptors [38]. In addition, nine distinct groups were obtained when evaluating 21 progenies of *C. canephora* half-brothers and with 14 morpho-agronomic traits considered [15], and seven groups (with a cut-off value of 33%) were found after evaluating the leaf morpho-anatomical traits of 34 *C. canephora* genotypes [20]. This indicates a higher variation in these genotypes than in the ones studied here.

The first group in the UPGMA tree was formed only by genotypes 10 and 37, which differ from the others due to their smaller number of epidermal cells and stomata, and they include the second largest polar diameter. Leaves with few but large stomata generally have lower maximum stomatal conductance than leaves with many but small stomata, due to a longer diffusion pathway within the leaf [42]. However, the genotypes showing fewer but larger stomata clearly display the shape of an ellipse (Figure 3, Table 3). The relationship between SL and SW is a good indication of the stomatal shape, since the stronger the relationship, the more elliptical the stomata will be, providing greater functionality due to less leaf transpiration, as confirmed by the high SLW values of these two genotypes. In contrast, the weaker the SL and SW relationship, the less elliptical and more near-circular the stomata will be, diminishing their functionality [22,60], which promotes changes in the physiological aspects of the plant. Higher densities of small stomata lead to increases in stomatal conductance to water vapor over the same total pore area, due to the shorter diffusion path length (45).

The second group was the largest in the UPGMA tree, comprising 22 genotypes. This group differs from the first one mainly because of a larger ECD. The third group consisted of seven genotypes with a somewhat greater ECD (the second highest) and smaller stomata (the lowest SL and SW). A reduced stomata size is an important trait related to gas exchange regulation. In addition, the difference in stomatal opening size has a greater effect on water diffusion than CO<sub>2</sub> diffusion [60,61]. For instance, stomatal size in *Banksia* leaves was found to be negatively correlated with the maximum rate of stomatal opening in response to light, showing that leaves with many small stomata exhibit faster water vapor conductance than their fewer, larger counterparts [62]. In fact, smaller stomata can respond faster than larger ones, thus increasing their function in dynamic environments [42].

The fourth group contained only genotypes 14 and 24, which had the highest SL and SW, as well as a high number of the largest stomata. In certain environmental conditions, exceptionally large stomata are not beneficial, as higher transpiration rates can occur during the opening process, which can have negative effects under water deficit conditions if the plant does not have other adjustment capabilities [63]. Variations in stomatal behavior, particularly regarding size, depend on the environment and the species genetic constitution, and these are commonly observed in plants under different stress conditions [61].

The fifth group was formed by genotypes with the third highest ECD, while the sixth group—which included genotypes 6, 19, 25, 21, and 26—had the highest ECD value. Both groups have intermediate-sized stomata. It is worth mentioning that the genotypes

integrating these two groups have high values of SD with a mean of 35 stomata, in relation to the high ECD, which must be considered in view of its significance for efficiency in the plant photosynthetic process, since the increase in SD may increase the CO<sub>2</sub> flow from the atmosphere into the leaf [41]. In addition, the higher SD of smaller stomata might have conditioned stomatal opening in a shorter time, thus reducing transpiration and providing better acclimation to low water availability conditions [61]. Therefore, SD is an important trait that directly affects leaf gas exchange [43]. In addition, greater stomatal density was observed in genotypes with resistance to water stress [60,63]. In fact, higher densities of small stomata lead to increases in stomatal conductance for the same total pore area, due to the shorter diffusion path length [45,63]. Although stomatal size and density contribute to global stomatal conductance, the control of the opening can override changes in morphology that are promoted by altered environmental conditions in coffee plants [62]. A negative relationship between stomatal size and functioning was observed between and within species [63]. Therefore, cultivars with smaller stomata are expected to exhibit faster responses.

From these results, we noted a wide diversity in the 43 genotypes studied. For example, the first and sixth groups have large differences in ECD, SL, and SW. Thus, the genotypes collected in more distant groups exhibited great dissimilarity and therefore can be promising for assisting in the selection of new plant materials.

#### 4.3. Correlation Studies of Biometric Variables

Correlation studies provide information about the strength of the associated traits [64]. The seven stomatal variables were either positively or negatively correlated with different levels of significance (Figure 3). In total, 21 relationships were estimated, of which 42.8% were classified as “very weak” (ranging from 0.00 to 0.19) according to the classification of Devore [65]. The correlations classified as “weak” (values between 0.20 and 0.39) [65] comprised approximately 19.2% of the total; these included ECD × SW (−0.23), ECD × SS (−0.37), ECD × SLW (−0.22) and ECD × SI (−0.31), all with  $p < 0.001$ .

Positive and negative correlations classified as “moderate” (0.40 to 0.69) [65] corresponded to 28.5% of the total; these included ECD × SL (−0.43), ECD × SD (0.40), SL × SLW (0.48), SW × SLW (−0.45), and SI × SD (0.61), all with  $p < 0.001$ . It is interesting to note that most correlations with ECD were significant and negative (except for SD). SLW had a positive and negative correlation with SL and SW, respectively. This behavior occurs because SLW is determined by the SL/SW ratio. A negative correlation between SW and SLW was also observed by Oliveira and Miglioranza [61] in cassava leaves. Strong positive correlations were found in the range of 0.70 to 0.89 [65] and corresponded to approximately 9.5% of the total.

Additionally, the morpho-anatomical characteristics that correlated with the production data of the genotypes of *C. canephora* showed two significant values, but they were considered low according to the Devore classification [66]. Stomata width (SW) had a value of 0.31 ( $p < 0.05$ ) and stomatal index (SI) returned a value of 0.34 ( $p < 0.05$ ). The stomatal index represents the investment of the plant in stomata production in relation to the total number of epidermal cells. This greater production conditions the plant to a higher rate of gas exchange, and wider stomata can facilitate this gas exchange. Both characteristics are favorable for the development and production of cultures, explaining the positive correlation observed in this study.

To complement the results of the correlations between anatomical leaf variables, we additionally performed a principal component analysis (PCA) (Figure 4). The first two components of this PCA explained 72.4% of the total variation, which can be considered quite accurate, since these two PCA components should concentrate the greatest amount of data variance in order to have divergence among genotype groups [20].

Based on the projection of the genotypes in the Cartesian plane defined by the two PCs, a considerable divergence was found regarding the evaluated characteristics, as reflected in their dispersion.

We observed the concentration of the variables regarding ECD, SD, and SI in PC1 negative and PC2. Therefore, SD is highly correlated because of the proximity in the positioning, since ECD and SI remain more distant, almost forming a 90° angle, evidencing a lower degree of correlation. The SLW, SL, SW, and SS traits were arranged in the positive quadrants of PC1 and PC2. It can be observed that the latter three factors are very close, evidencing a strong relationship, since the estimates of SLW and SS are based on the linear measurements of the stomata.

A positive relationship between increases in SD and tree height was found when examining the leaf morpho-anatomical traits of 35 rainforest tree species in Central Amazonia [43]. However, that study determined that the regression equation could only explain 14% of the variance in SD; therefore, other factors are likely involved in the determination of SD.

## 5. Conclusions

A strong morphological divergence was found between the 43 *C. canephora* genotypes based on seven leaf morpho-anatomical traits. The UPGMA grouping method clustered the genotypes into six groups. Genotypes 10 and 37 showed a higher dissimilarity than the other genotypes. Leaf traits showed different levels of correlation, with higher results between stomatal length and stomatal width, index, and density. The phenotypic variation in the evaluated characteristics was predominantly due to genetic causes. The correlation and principal component analyses indicated the characteristics with a higher degree of correlation. The genotypes show potential for future plant breeding purposes, and this can assist in the choice of more promising plant materials.

Considering the results obtained in this study, we reinforce that diversity is a prerequisite for the breeding of agricultural crops. In addition, characterizing the regional diversity of a crop is important for understanding and directing specific improvements in a specific location. The traits studied in the present work are of great interest in the context of changes since they can be exploited in breeding cultivars to adapt the *C. canephora* crop to a broader range of environmental conditions.

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