



# Article Growth, Physiological, Nutrient-Uptake-Efficiency and Shade-Tolerance Responses of Cacao Genotypes under Different Shades

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Abstract: Cacao is an understory plant cultivated in full-sun monocultures, multistrata agroforestry systems, where cacao trees are planted together with fruit, timber, firewood, and leguminous trees, or within thinned native forests. In agroforestry systems of cultivation, cacao is subjected to excess shade due to high density, excess growth, and the unmanaged pruning of shade trees. Cacao is tolerant to shade, and the maximum photosynthetic rate occurs at an irradiance of around 400  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>. However, excess shade further reduces the irradiance, which is detrimental to photosynthesis and growth functions. Intra-specific variation is known to exist in cacao for the required saturation irradiance. A greenhouse study was implemented with 58 cacao genotypes selected from four geographically diverse groups: (i) wild cacao from river basins of the Peruvian Amazon (PWC); (ii) Peruvian farmers' collection (PFC); (iii) Brazilian cacao collection (BCC); and (iv) national and international cacao collections (NIC). All of the cacao genotypes were subjected to 50% and 80% shade where photosynthetic photon flux density (PPFD) was 1000 and 400  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, respectively. Intra-specific variations were observed for growth, physiological and nutritional traits, and tolerance to shade. Cacao genotypes tolerant to shade were: UNG-77 and UGU-130 from PWC; ICT-2173, ICT-2142, ICT-2172, ICT-1506, ICT-1087, and ICT-2171 from PFC; PH-21, CA-14, PH-990, and PH-144 from BCC; and ICS-1, ICS-39, UF-613, and POUND-12 from NIC. Genotypes that tolerate excess shade may be useful plant types for maintaining productivity and sustainability in agroforestry systems of cacao management.

Keywords: Theobroma cacao; light; abiotic stress; physiology; plant nutrition

# 1. Introduction

In addition to the basic concept of shade tolerance, which is defined as the minimum light required for a plant to survive and develop in different strata, this factor involves a wide range of effects that are connected to several aspects of the plant life cycle and ecosystem dynamics [1–4]. The adaptation of species to shade is still poorly understood [5], and the positive or negative effects are sometimes contradictory, which is the case in cacao [6].

Cacao (*Theobroma cacao*) is an understory plant [7,8] cultivated under different cropping systems: from full-sun monocultures to multistrata agroforestry systems, where cacao trees are planted together with fruit, timber, firewood, and leguminous trees, or within thinned



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**Copyright:** © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). forests [7,8]. The use of shade trees is a common agricultural and sustainable practice in cacao production systems [9–16]. These trees act as a protection barrier against stressful environmental conditions, such as extreme temperature, solar radiation, drought, and intense rainfall, and wind [6,7,17,18].

Several benefits have been attributed to cacao growing within shaded agroforestry systems, such as control of diseases or insect attacks, maintaining soil fertility, enhancing nutrient cycling, reducing soil erosion and deforestation, increasing tree diversity, mitigating climatic changes thorough C sequestration, and helping to reduce the use of pesticides and fertilizer applications [13,19–26].

However, some authors have reported that lower yields have been observed in shaded cacao systems, mostly related to climate conditions (i.e., precipitation and temperature) and probably because, in these systems, the lower light reduce the photosynthetic activities needed to better yields [27–30]. Nonetheless, farmers can achieve higher net revenues because of the lower maintenance cost (compare to conventional systems) and higher prices obtained per kilogram of cacao beans [31].

Cacao is considered a sun species that is tolerant to moderate shade [32]. Its yield is affected by the presence of too many trees, trees with large canopies, and poorly managed tree canopy structures [24,33]. Under these conditions, the longevity of the plantation may also be affected [34]; therefore, it is advantageous to adopt cacao genotypes that maintain their productivity despite high shade levels, and that can be recommended for the establishment of agroforestry systems.

The definition of shade levels in cacao is a major concern, especially for agroforestry systems [7,17,34], as well as the selection of appropriate tree species to avoid the detrimental effects of shade [6]. It is also important to mention that the need for shade may not be required in all cacao-growing regions, especially in island and heavy cloud cover ecosystems [6].

In tropical forests, understory plants receive a photosynthetic photon flux density (PPFD) of between 5 and 25  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> or 1 to 2% of the irradiance obtained above the tree canopy level but they also intermittently obtain high levels of PPFD in the form of sunflecks [35–41]. Miyaji et al. [42] reported that the light intensity (at full daylight) above the cacao canopy shaded by trees in Bahia, Brazil was between 30% and 100%, and between 4 and 10% at ground level. In another study conducted in Alto Beni (Bolivia), Niether et al. [18], measured the light levels of various cacao systems and determined a PPFD between 1580 and 2028  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> above the canopy. In contrast, in an open sun monoculture, irradiance varied between 985 and 1546  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> depending on canopy pruning (before and after, respectively). Consequently, light levels have significant influence on growth, physiological traits and nutrition of cacao [33,43,44].

It has been reported that maximum photosynthesis in cacao occurs at a PPFD of 350 to 550  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, which is about 20% to 25% of the intensity of full sunlight [44–47]. In some cacao genotypes, an increase in PPFD from 50 to 400  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> entails an increase in the net photosynthetic rate (P<sub>N</sub>) by about 50%, but further increases (up to 1500  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) have no effect, indicating that very little radiant energy is required to support efficient P<sub>N</sub> in cacao [43].

Variations in morphological characteristics among different genotypes have been reported in cacao [48,49]. In addition, these characteristics are influenced by the level of irradiance [10,11,43,50]. Baligar et al. [43] reported that, in juvenile cacao genotypes, increasing PPFD from 65 to 190  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> increased shoot and root growth, mineral nutrition, and net assimilation rate. Thus, the identification of plant traits for growth and physiological parameters that are influenced by light quality and intensity (PPFD) will help to identify cacao genotypes that may perform well under a range of light intensities (or shade levels).

This research aimed to evaluate the effects of two levels of shade (50% and 80%) on growth, physiological, macronutrients, and micronutrients use efficiency responses of national (wild, domesticated) and international cacao genotypes.

## 2. Materials and Methods

#### 2.1. Experimental Conditions

## 2.1.1. Cacao Genotypes

The greenhouse experiment was implemented at the Instituto de Cultivos Tropicales-ICT (Tropical Crops Institute), Tarapoto, San Martin, Peru. A total of 58 cacao genotypes from the germplasm bank located at "El Choclino" experimental station was selected from four geographic origins: (i) wild cacao from river basins of the Peruvian Amazon collection (PWC); (ii) Peruvian farmers' collection (PFC); (iii). Brazilian cacao collection (BCC); and (iv) national and international cacao collections (NIC). Several expeditions were undertaken by ICT during 2008 to collect wild cacao genotypes from the river basins of Aypena (AYP), Pastaza (PAS), Ungumayo (UGU), and Ungurahui rivers (UNG). Peruvian farmers' collections were gathered by ICT from 2002 to 2004 in the Provinces of Mariscal Cáceres and Tocache of San Martin department in northeast Peru. Brazilian clones were sourced from Comissão Executiva do Plano da Lavoura Cacaueira/Centro de Pesquisas de Cacau (CEPLAC/CEPEC), Ilhéus/Itabuna, Bahia, Brazil; and international clones were sourced from CFC/ICCO/ Bioversity Clones, International Cocoa Quarantine Centre, University of Reading, Reading, UK (Table 1).

Table 1. List of 58 cacao genotypes used for this experiment from 4 different origins maintained at the ICT germplasm bank.

Wild Cacao Genotypes from River Basins of Peru Amazon (PWC)				Peruvian Farmers' Cacao Genotypes (PFC)			Brazilian Gen	otypes (BCC)	National and International Genotypes (NIC)		
N°	Genotype	Origin	N°	Genotype Origin N° Genotype Origin		Origin	N°	Genotype	Origin		
1	AYP-15	Aypena	1	ICT-1026	Mariscal Cáceres—Juanjui	1	BN-34	Fazenda Boa Nova	1	CCN-10	Ecuador
2	AYP-20	Aypena	2	ICT-1087	Mariscal Cáceres—Juanjui	2	BS-01	Fazenda Bom Sossego	2	CCN-51	Ecuador
3	AYP-22	Aypena	3	ICT-1092	Mariscal Cáceres—Juanjui	3	CA-14	Fazenda Canta Galo	3	EET-400	Ecuador
4	PAS-91	Pastaza	4	ICT-1112	Mariscal Cáceres—Juanjui	4	CEPEC-2002	Centro de pesquisa do cacau	4	H-10	Peru
5	PAS-93	Pastaza	5	ICT-1189	Mariscal Cáceres—Juanjui	5	CP-49-C10	Centro de pesquisa do cacau	5	ICS-1	Trinidad and Tobago
6	PAS-100	Pastaza	6	ICT-1251	Mariscal Cáceres—Juanjui	6	CP-53-C10	Centro de pesquisa do cacau	6	ICS-6	Trinidad and Tobago
7	PAS-105	Pastaza	7	ICT-1281	Mariscal Cáceres—Juanjui	7	IPIRANGA-1	Cidade de Ipiranga	7	ICS-39	Trinidad and Tobago
8	UGU-112	Ungumayo	8	ICT-1292	Mariscal Cáceres—Juanjui	8	PH-09	Fazenda Porto Hibrido	8	ICS-95	Trinidad and Tobago
9	UGU-126	Ungumayo	9	ICT-1506	Mariscal Cáceres—Juanjui	9	PH-15	Fazenda Porto Hibrido	9	IMC-67	Peru
10	UGU-130	Ungumayo	10	ICT-2142	Tocache	10	PH-16	Fazenda Porto Hibrido	10	POUND- 12	Peru
11	UNG-53	Ungurahui	11	ICT-2161	Tocache	11	PH-17	Fazenda Porto Hibrido	11	SCA-6	Peru
12	UNG-73	Ungurahui	12	ICT-2171	Tocache	12	PH-21	Fazenda Porto Hibrido	12	TSH-565	Trinidad and Tobago
13	UNG-76	Ungurahui	13	ICT-2172	Tocache	13	PH-144	Fazenda Porto Hibrido	13	TSH-1188	Trinidad and Tobago
14	UNG-77	Ungurahui	14	ICT-2173	Tocache	14	PH-990	Fazenda Porto Hibrido	14	UF-613	Costa Rica
			15	ICT-2653	Tocache			Thered	15	UF-667	Costa Rica

## 2.1.2. Shade (PPFD) Levels

The greenhouse was constructed and aligned in an east–west direction to evaluate the response of cacao genotypes to two levels of shade (Figure 1). The greenhouse was divided into two sections and covered with different light transmissibility plastic screens (Raschel mesh, Arborizaciones EIRL<sup>®</sup>) to achieve different levels of shade. The first section provided 80% shade with a PPFD of 400  $\pm$  50 µmol m<sup>-2</sup> s<sup>-1</sup>, and the second section provided 50% shade with PPFD of 1000  $\pm$  50 µmol m<sup>-2</sup> s<sup>-1</sup>. The PPFD in each section of the greenhouse was measured by a MQ-200 Quantum sensor (Apogee Instruments, Logan, UT, USA). At both ends of the greenhouse, exhaust and inlet fans were installed to circulate air from inside to outside.



**Figure 1.** Greenhouse constructed at the Tropical Crops Institute (ICT), Tarapoto, Peru to evaluate the response of 58 cacao genotypes to two levels of shade: 50% (PPFD of  $1000 \pm 50 \text{ }\mu\text{mol }\text{m}^{-2} \text{ s}^{-1}$ ) and 80% (PPFD of  $400 \pm 50 \text{ }\mu\text{mol }\text{m}^{-2} \text{ s}^{-1}$ ).

#### 2.1.3. Plant Growth Conditions

The rooted clonal cuttings from plagiotropic branches of various genotypes were prepared in the greenhouse. Terminal apical shoots with 3 or 4 leaves from each of the genotypes were cut, by making a bevel cut, at the base of the branches (3/4 from the leaf area) and dipped into plant rooting hormone (Hormodin3<sup>®</sup>, 0.8% indole-3-butyric acid, IBA) to induce roots. These cuttings were transplanted into polyethylene bags containing 2 kg of agricultural soil (sand = 50.96%, silt = 22%, clay = 27.04%) previously fertilized with 60 N:50P:90 K kg ha<sup>-1</sup> applied as urea, calcium dihydrogen phosphate, and potassium chloride, respectively. Dolomitic lime (1 MT  $ha^{-1}$ ) was added to raise the pH to 6.0. Plants were maintained in the greenhouse at 28 °C, 80% relative humidity, with minimum light PPFD 50  $\pm$  5 µmol·m<sup>-2</sup> s<sup>-1</sup>; soil moisture was maintained at the field capacity until root formation and proper pest control methods were adapted. At the end of 4 months' growth, seedlings were transplanted into plastic pots containing 5 kg of sandy loam soil previously fertilized with urea (30 mg N kg<sup>-1</sup>), Ca(H<sub>2</sub>PO<sub>4</sub>)<sub>2</sub> (25 mg P kg<sup>-1</sup>), KCl (45 mg K kg<sup>-1</sup>), and dolomitic lime (2.5 g kg<sup>-1</sup>). Soil physicochemical properties of the sandy loam soil used for the study were: 72% sand, 18% clay, 1.55 g cm<sup>-3</sup> bulk density, 58% porosity, pH 6.1, organic matter 1.77%, CEC 4.44 cmol kg<sup>-1</sup>, N (0.08%), P (4 mg kg<sup>-1</sup>), K (75 mg kg<sup>-1</sup>), Ca (1.22 cmolc<sup>+</sup> kg<sup>-1</sup>), and Mg (0.60 cmolc<sup>+</sup> kg<sup>-1</sup>), determined using the methods of Silva [51].

All the rooted genotype seedlings were divided into two equal groups and subjected to two shade (PPFD) levels (Figure 1). Plants were grown for 6 months under different shade levels, and soil moisture during growth was maintained at field capacity (-33 KPa) by watering with deionized water every other day; soil moisture status was monitored with a soil moisture tensiometer (2724 ARL Jet Fill tensiometer, Soilmoisture Equipment Corp, Santa Barbara, CA, USA). During growth of the plants, the greenhouse provided a mean temperature of 30.0 °C and relative humidity of 63%. The experiment was conducted using a split-plot design with three replications under complete random distribution; two shade treatments were the main plots (50% and 80% shade) and 58 cacao genotypes were the subplots.

## 2.2. Biometric Parameters

At the time of harvest, shoot length was measured from the base of the stem to the apex of the plant (cm) with a ruler, and stem diameter was measured using a digital Vernier (mm), respectively. At harvest, stems, leaves, and roots were separated; leaf and root area were measured in cm<sup>2</sup> using image analysis (Assess 2.0: Image Analysis Software for Plant

Disease Quantification, APS, Saint Paul, MN, USA) [52]. All plant parts were washed with tap water, dipped in 1% HCl, and rinsed in distilled water, placed in paper sachets, and oven-dried at 60 °C for 72 h, until reaching a constant weight.

## 2.3. Physiological Parameters

A week before harvest, three mature leaves were selected randomly per genotype/pot and per shade treatment to record stomatal conductance (in mmol  $m^{-2} s^{-1}$ ) using a SC-1 leaf porometer (Decagon Devices, Pullman, WA, USA). Leaf chlorophyll content or "greenness" (in SPAD units) was measured using a SPAD 502-Plus ((Konica Minolta, Inc., Tokyo, Japan).

Water use efficiency (WUE, g plant<sup>-1</sup> L<sup>-1</sup>) was calculated as follows:

$$WUE = \frac{SDW}{\text{Total water used during entire growth}}$$
(1)

where SDW = shoot dry weight, g plant<sup>-1</sup>

Total water used during entire growth period = 18.0 L (50% shade) and 11.7 L (80% shade) plant<sup>-1</sup>.

# 2.4. Nutrient Uptake Parameters

## 2.4.1. Concentration of Nutrients

Oven-dried shoots of all genotypes were ground to pass through a 1 mm sieve and 500 mg of sample was digested in 10 mL of 65% HNO<sub>3</sub> [53]. The concentrations of macronutrients (K, Ca, Mg) and micronutrients (Cu, Fe, Mn, and Zn) in the digested material were determined by atomic absorption spectrophotometry (AAS, Varian model "Spectra 55B", Victoria, Australia); P was determined using the ascorbic-molybdate color development method [51]. Total N was determined by the Kjeldahl method (digestion with 5 mL of H<sub>2</sub>SO<sub>4</sub> 95%), and both N and P were detected using a spectrophotometer (Spectronic 20D, Thermo Fisher, Waltham, MA USA) [51]. Concentrations are presented as the mean values from three replicates and expressed in g kg<sup>-1</sup> for macronutrients and mg kg<sup>-1</sup> for micronutrients.

# 2.4.2. Nutrient Uptake (U)

The nutrient uptake (U) or element content was calculated as follows:

$$U = \frac{\text{Element concentration } \times \text{SDW}}{1000}$$
(2)

where

SDW (shoot dry weight) = in g plant<sup>-1</sup>

Element concentration = in g kg<sup>-1</sup> (for macronutrients) or mg kg<sup>-1</sup> (for micronutrients) U (or content) = g plant<sup>-1</sup> (macronutrients) or mg plant<sup>-1</sup> (micronutrients)

# 2.4.3. Nutrient Uptake Efficiency (NUE)

Nutrient uptake efficiency (NUE), which is used to differentiate plant species, genotypes, and cultivars based on their ability to absorb and utilize nutrients for maximum yields [54,55], was calculated as follows:

$$NUE = \frac{1}{\text{Element concentration}} \times 1000$$
(3)

where

element concentration =  $g kg^{-1}$  for macronutrients and  $mg kg^{-1}$  for micronutrients NUE = in g shoot  $g^{-1}$  of any macronutrient or in g shoot  $mg^{-1}$  of any micronutrient

## 2.5. Shade Tolerance Index (STI)

Shade tolerance is the ability of a tree to survive and develop under light-limited conditions [2,3]. In this study we consider cacao genotype that does not or shows little decrease in total biomass when grown in low light (high shade) conditions is considered shade-tolerant and to classify the tolerance of cacao genotypes, STI was calculated as described in the following equation:

$$STI = \frac{\text{Totol dry biomass at 80\% shade}}{\text{Total dry biomass at 50\% shade}} \times 100$$
(4)

where

total (shoot + root) dry biomass (g plant<sup>-1</sup>) at 80% shade (PPFD 400 µmol m<sup>-2</sup> s<sup>-1</sup>); this represents low light or heavy shade;

total (shoot + root) dry biomass (g plant<sup>-1</sup>) at 50% shade (PPFD 1000  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>); this represents high light or low shade.

Genotypes were classified into 3 groups: sensitive to shade (STI%  $\leq$  40), medium shade-tolerant (40 < STI%  $\leq$  60), and tolerant to shade (STI% > 60).

## 2.6. Statistical Analysis

Statistical analyses of all of the parameters were performed using Infostat ver. 2020 [56] and consisted of a split-plot design under complete random distribution to compare means across two or more independent variables; in this case, shade was the main plot and cacao genotypes were subplots. The normality and homogeneity of each parameter were determined using the Shapiro–Wilk test and Q-Q plot. When the effect of interactions between factors was not statistically significant ( $\alpha > 0.05$ ) and the effect of a factor was significant, the analysis was extended to include a Scott–Knott test to compare means of the parameters.

Double bar graphs were drawn for NU and NUE, where the results of each nutrient are represented with comparisons between treatments: Shade—50% and Shade—80%, the average for each origin. For NUE, the *x*-axis is presented in a logarithmic scale to facilitate the interpretation of the results. "ns" indicates the results were not significant (p > 0.05).

Principal Component Analysis (PCA) using R software version 1.2.5042 was used to evaluate the general correlations between standardized data of biometric, physiological parameters, and nutrient uptake (NU) of cacao seedlings for genotypes sensitive to shade and tolerant to 50% and 80% shade. The objective of this analysis was also to assess differences in nutrition that would partially explain the mechanisms of shade tolerance in cacao. In this analysis, two principal components were used as they accounted for more than 50% of the variance of all variables for both 50% and 80% shade treatments.

## 3. Results and Discussion

Plants can adjust their morphology and physiology to a particular light environment. In the case of tropical and subtropical tree species, they have developed species-specific morphological and physiological features that allow them to optimize the capture of scarce solar radiation [37]. Larger leaf areas with anatomical properties associated with increased photosynthetic efficiency, in addition to an accumulation of anthocyanin, are some of the responses of trees to low and high irradiance levels [37,50] whereas root dry matter remains invariable, suggesting few anatomical or chemical changes [57].

Several authors have reported negative effects of shade on cacao growth [30,58], whereas greater chlorophyll contents [7] and higher photosynthetic rates related to water regime have been observed in plants under light shade, thus highlighting the genotypic differences as a response to shade [9,59]. The following sections summarize the results obtained in this study for 58 cacao genotypes subjected to 50% and 80% shade conditions.

## 3.1. Growth Parameters Influenced by Shade Levels

A lot of the growth parameter responses of cacao under different light regimes reported here are quite similar to most plants as well as a lot of the cacao literature cited (Table 2 and Table S1). The interactions between cacao genotypes and shade levels for biometric parameters were significant ( $p \le 0.05$ ), except for leaf area (Table 2); however, for this parameter, significant differences were found between the shade levels and across genotypes (Table 2 and Table S1). These findings agree with those of a previous study conducted by Daymond et al. [60], in which leaf area varied significantly in eight cacao clones (AMAZ 15/15, ICS-1, IMC-47, MAN 15/2, SC-1, SCA-6, SPEC 54/1, UF-676) exposed to different levels of irradiance (0 to 696 µmol m<sup>-2</sup> s<sup>-1</sup>). As highlighted by Acheampong et al. [9], genotypic differences in leaf area under different shade levels suggest differential partitioning of assimilates in response to light in terms of final biomass.

Table 2. Effect of shade on growth parameters in 58 cacao genotypes from different origins subjected to 50% and 80% shade.

							Source of Variation [df]			
Growth Parameter *	Unit per Plant	PWC	PFC	ВСС	NIC	Total	Shade (S) [1]	Genotype (G) [57]	G*S [57]	
								pv		
SL 50%	cm	33.62	39.56	34.56	39.05	36.79	0.0004	< 0.0001	0.0072	
SL 80%	cm	44.60	51.90	42.46	45.30	46.15				
SL $\Delta$	cm	-10.98	-12.34	-7.90	-6.25	-9.36				
SD 50%	mm	10.17	12.14	9.80	12.04	11.07	0.0001	< 0.0001	0.0003	
SD 80%	mm	7.88	9.27	8.06	9.03	8.58				
SD $_{\Delta}$	mm	2.29	2.87	1.74	3.01	2.49				
LA 50%	cm <sup>2</sup>	780.59	898.51	834.11	952.53	868.47	0.0001	< 0.0001	0.6657	
LA 80%	cm <sup>2</sup>	1023.99	1145.91	1037.56	1188.25	1110.27				
LA $\Delta$	cm <sup>2</sup>	-243.40	-247.40	-203.45	-235.72	-241.80				
RA 50%	cm <sup>2</sup>	241.50	300.99	317.97	327.91	297.69	< 0.0001	< 0.0001	0.0008	
RA 80%	cm <sup>2</sup>	295.97	388.69	337.21	418.28	361.54				
$RA_{\Delta}$	cm <sup>2</sup>	-54.47	-87.70	-19.24	-90.37	-63.85				
RDW 50%	g	6.94	10.61	7.21	9.02	8.50	< 0.0001	< 0.0001	< 0.0001	
RDW 80%	g	2.38	3.93	2.92	3.60	3.23				
RDW $\Delta$	g	4.56	6.68	4.29	5.42	5.27				
BDW 50%	g	30.81	35.58	30.05	32.08	32.19	< 0.0001	< 0.0001	< 0.0001	
BDW 80%	g	13.83	18.76	14.43	17.16	16.11				
BDW $\Delta$	g	16.98	16.82	15.62	14.92	16.08				
S/R 50%		3.92	2.44	3.45	2.64	3.09	0.0001	< 0.0001	0.0006	
S/R 80%		5.53	4.01	4.23	3.99	4.42				
$S/R_{\Delta}$		-1.61	-1.57	-0.78	-1.35	-1.33				

\* SL: shoot length; SD: stem diameter; LA: leaf area; RA: root area; RDW: root dry weight; BDW: total dry weight; S/R: shoot/root rate; PWC: wild cacao (from river basins of Peruvian Amazon) [n = 14]; PFC: Peruvian farmers' cacao [n = 15]; BCC: Brazilian cacao [n = 14]; NIC: national and international cacao collections [n = 15].

In most cases, mean values of shoot length, leaf area, root area, and shoot/root ratio per genotype were higher under 80% shade, and stem diameter, root dry weight, and total dry biomass were higher under 50% shade (Table 2 and Table S1). The slow growth of the length of the shoots at higher light levels is probably due to the limitations in the expansion of the leaves caused by greater transpiration; on the contrary, in low light conditions the greater growth is due to the plants seeking light [7], stem diameter increases under high light environments because the shoots are not growing as tall, and leaf area increases in low light in an attempt to capture more light [7,61]. The minimum values of leaf and root area were observed for the AYP-20 genotype at 50% and 80% shade. The lowest value of stem diameter, of 6.45 mm (80% shade), was recorded for the UGU-130 genotype (Table S1). The minimum values of shoot length were 23.07 cm (50% shade) and 33.07 cm (80% shade) for PH-09 and CA-14 (BCC), respectively (Table S1).

As reported by Galyuon et al. [12], cacao grown under full sunlight (1800  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) and shade (900  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) had different morphologies. Under full sunlight, leaf size, internode length, total leaf area, and dry matter per plant were significantly reduced, whereas leaf thickness and leaf number per plant were increased compared to plants grown in shade. In addition, cacao plants grown between 35% and 55% shade have a leaf area higher than those grown under heavy shade, whereas stem dry matter accumulation decreases as the level of shade increases [9].

Da Silva Branco et al. [10] evaluated the effects of four levels of shade (no shade, 50% 29%, and 22% of the incidence of radiation) and two levels of water regime (control and flooded plants) on cacao seedlings' performance. They reported that responses of shoot length, stem diameter, leaf number, total leaf area, specific leaf area, root biomass, and total biomass varied by genotype, treatment, or combined effects. For example, leaf number and leaf area were reduced by increasing light intensity; by comparison, root length and collar diameter did not vary when exposed to different shade levels.

Similarly, stem diameter was found to be greater in cacao monocultures, followed by agroforestry and successional agroforestry systems, in a long-term field trial in Alto Beni, Bolivia [18]. Nevertheless, the production system (full-sun monoculture, agroforestry system, both under organic and conventional farming, and a highly diverse successional agroforestry system under organic farming) did not affect root length, surface area, specific root length, specific root area, or diameter [62]. However, in the highly successional agroforestry system, root volume and biomass were higher than those found in the agroforestry system [62]. These results are in agreement with our findings (Table 2). An investment in root growth is most relevant in plants under high rather than low light intensity to provide a large surface for evapotranspiration and to maintain cell turgor pressure [10].

A maximum shoot/root ratio was recorded for the AYP-15 cacao genotype (PWC) for both shade levels. The highest values of root dry weight, of 12.65 and 5.70 g plant<sup>-1</sup>, were noted for the genotypes ICT-1112 (PFC) and CCN-51 (NIC) at 50% and 80% shade, respectively. The genotypes having the highest total dry weight were PAS-91, 42.38 g plant<sup>-1</sup> (50% shade), and ICT-1087, 26.77 g plant<sup>-1</sup> (80% shade) (Table S1). Baligar et al. [43] observed an increase in biomass accumulation in cacao roots, leaves, stems, and shoots in a greenhouse experiment when PPFD increased from 65 to 190 µmol m<sup>2</sup> s<sup>-1</sup> (high to medium shade), which also entailed a reduction in leaf area and leaf specific area. Recently, Baligar et al. [61] reported that, in seven genetically different cacao genotypes, increasing PPFD from 100 to 400 µmol m<sup>-2</sup> s<sup>-1</sup> increased shoot and root growth, relative growth rate (RGR), and net assimilation rate (NAR). Moreover, cacao grown under two shaded systems, one with N-fixing legume trees and one with different shade trees, compared to a monoculture system, had higher stem density, shoot:root ratio, and above-ground and root biomass, but a similar tree height and a lower stem diameter [19].

#### 3.2. Physiological Parameters Influenced by Shade

The interaction between cacao genotypes and shade levels was significant for the physiological parameters (Table 3).

An increase in chlorophyll content (SPAD unit) was observed in cacao seedlings under 80% shade compared to 50% shade, with some exceptions across genotypes. By contrast, overall stomatal conductance was higher in plants grown under 50% shade; however, there were some exceptions in genotypes of PFC and NIC (Table 3).

The maximum level of chlorophyll content at 50% shade was 34.70 SPAD for the UGU-130 genotype, whereas at 80% shade it was 34.50 SPAD for AYP-15; both were from PWC. The range of observed stomatal conductance was between 104.57 and 363.03 mmol m<sup>-2</sup> s<sup>-1</sup> at 50% shade for UGU-112 and PH-21, respectively, and between 47.33 and 260.67 mmol m<sup>-2</sup> s<sup>-1</sup> at 80% shade for UGU-126 and H-10, respectively (Table S2).

							Source of Variation [df]		
Physiological Parameter *	Unit	PWC	PFC	BCC	NIC	Total	Shade (S) [1]	Genotype (G) [57]	G*S [57]
								pv	
CHL 50%	SPAD	33.74	29.17	31.71	28.66	30.75	< 0.0001	< 0.0001	< 0.0001
CHL 80%	SPAD	33.55	30.44	33.46	31.19	32.12			
CHL $\Delta$	SPAD	0.19	-1.27	-1.75	-2.53	-1.37			
$\mathrm{gs}$ 50%	$ m mmol~m^{-2}~s^{-1}$	188.40	184.01	226.86	183.78	195.35	0.0001	0.0002	0.0007
gs <sub>80%</sub>	$ m mmol\ m^{-2}\ s^{-1}$	70.56	169.41	115.04	139.80	124.77			
gs $\Delta$	$ m mmol\ m^{-2}\ s^{-1}$	117.84	14.60	111.82	43.98	70.58			
WUE 50%	$ m g \ L^{-1}$	1.33	1.39	1.27	1.28	1.31	0.0003	< 0.0001	< 0.0001
WUE 80%	$\tilde{g}$ L <sup>-1</sup>	0.98	1.27	0.98	1.16	1.09			
WUE $\Delta$	$\widetilde{g}$ L <sup>-1</sup>	0.35	0.12	0.29	0.12	0.22			

Table 3. Effect of shade on physiological parameters in 58 cacao genotypes from different origins Scheme 50 and 80% shade.

\* CHL: leaf chlorophyll content; gs: stomatal conductance; WUE: water use efficiency; PWC: wild cacao (from river basins of Peruvian Amazon) [n = 14]; PFC: Peruvian farmers' cacao [n = 15]; BCC: Brazilian cacao [n = 14]; NIC: national and international cacao collections [n = 15].

Concerning chlorophyll content, shade adapted plants tend to have greater concentrations of chlorophyll to capture more light [32]. Shade leaves in cacao often exhibit greater total chlorophyll concentrations per unit mass than sun leaves [32]. Almeida and Valle [63] and Daymond and Hadley [59] reported a consistent decline in chlorophyll content with increasing light in three cacao genotypes, and this factor can vary considerably over time. Moreover, Acheampong et al. [64] reported that no clear pattern was observed for leaf chlorophyll in cacao genotypes grown under three shade levels provided by plants and under fertilizer application. Our results show either a reduction in leaf chlorophyll content as PPFD increases or little variation between shade levels (Table 3 and Table S2).

Acheampong et al. [9] conducted a study with four cacao genotypes (T 79/501, PA-150, SCA-6, and P-30) and subjected them to three shade levels (32.5%, 55%, and 76%) during two seasons of growth (dry and rainy). They reported that stomatal conductance was higher in seedlings under heavy shade during the dry season, and lower in the rainy season for seedlings under lighter shade. As a consequence, photosynthesis rates were higher in the wet season for cacao under medium and light shade. The results from one crop year for the same genotypes grown under three shade levels provided by plants and under fertilizer application showed that a higher stomatal conductance was related to an increase in shade, but only for two genotypes [64]. However, the opposite trend was observed in our study, in which stomatal conductance decreased with higher shade (Table 3 and Table S2); this was shown by the overall results and specific values for the SCA-6 cacao genotype (Table S2). Overall stomatal conductance was higher in plants grown at low shade (50% shade); however, there were some exceptions in genotypes of PFC and NIC (Table S2).

The evaluation of stomatal conductance in nine cacao genotypes (TCS-13, TCS-19, SCC-53, SCC-82, SCC-83, CCS-73, CCS-77, CCS-80, and ICS-95) grown in two agroforestry systems in Colombia with a maximum irradiance of 2100 and 1800  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> showed lower stomatal conductance for the former, due to a lower transpiration rate associated with low water bioavailability in the soil [65]; this is in alignment with our overall results.

Nevertheless, if we only consider the ICS-95 genotype, a slight increase in stomatal conductance was observed when exposed to a higher PPFD (Table S2). Da Silva Branco et al. [10] found that a decrease in stomatal conductance in TSA-792 and TSH-774 cacao genotypes was observed when plants were subjected to flooding, but not when the light intensity was attenuated. These results were attributed to an accumulation of abscisic acid (ABA) with the increase in shading density, which regulated the stomatal opening. The opposite was observed in our study, in which a decrease in stomatal conductance in TSH cacao genotypes was recorded when seedlings were exposed to a higher shade level (Table S2).

Under field conditions, Jaimez et al. [66] reported that an increase in PPFD (from 400 to 1000  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) in a 7-year-old cacao plot in Ecuador involved an increase in net

photosynthetic rate, while high stomatal conductance was maintained, although in some cases greater stomatal conductance at a low PPFD (high shade) was recorded for some genotypes. Baligar et al. [44] reported that stomatal conductance (around 0.02 mol m<sup>-2</sup> s<sup>-1</sup>) in three cacao genotypes (CCN-51, LCT EEN 37/A, and VB 1117) was not significantly affected by PPFD in the range of 50 to 400  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>. Nonetheless, maintaining a high conductance at very low irradiance may be an advantage to understory plants, by allowing photosynthesis to respond rapidly to sunflecks.

When cacao leaves were continually exposed to light intensities higher than half of that in which instantaneous maximum photosynthesis occurred (about 350–400 µmol m<sup>-2</sup> s<sup>-1</sup>, which is nearly 20% of the intensity of full sunlight), the rate of photosynthesis began to decline after four hours. At light intensities higher than 100% of saturating photosynthetic intensity, the decline began almost immediately, causing a certain degree of photoinhibition [39]. Taking into account these findings, our results indicate that shade levels of 50% (PPFD of  $1000 \pm 50 \text{ µmol m}^{-2} \text{ s}^{-1}$ ) may be too high and may cause some form of stress in terms of chlorophyll content, whereas the increase in stomatal conductance was probably due to an increase in leaf temperature under 50% shade.

In this study, WUE was significantly affected by shade, genotypes, and their interaction. WUE tended to decrease significantly with the increase in shade (Table 3). The average observed WUE under 50% shade was 1.32 g  $L^{-1}$ , which was significantly higher than the value of  $1.12 \text{ g L}^{-1}$  obtained under 80% shade (Table S2). Similar tendencies were reported by Lopez-Marin et al. [67], with a negative correlation between shade levels and WUE in a greenhouse experiment with sweet pepper plants, in which the highest values were registered under non-shaded conditions. In addition, Yang [68] found similar tendencies in forages. In cacao growing under high and low PPFD, Jaimez et al. [66] found that WUE showed a negative linear relationship with light level. In addition, the high WUE observed in the evaluated clones under 50% shade (Table 3 and Table S2) indicated the possibility of growing these cacao genotypes with less shade. In contrast, in a study of coffee in the dry season, Baliza et al. [69] found WUE increased as shade increased to 50%, before decreasing under higher levels of shade. In the current study, the maximum levels of WUE, of 1.77 g  $L^{-1}$ and 1.81 g  $L^{-1}$ , were recorded for the ICT-1087 genotype (PFC) under 50% and 80% shade, respectively. The high WUE recorded in this genotype could be related to its overall highest shoot and root dry biomass under both levels of shade (Table S2).

### 3.3. Nutritional Status Influenced by Shade

Numerous effects of shade on cacao are not well understood, including the differences in response to specific nutrients under shade [26]. Some cacao varieties are more nutrient demanding than others [61]. In soils with low fertility, shade acts as a buffer that reduces metabolic activity, which also reduces nutrient uptake. Cacao trees without shade demand higher quantities of nutrients than shaded trees: the former may contain higher levels of N and K than the latter, which have higher levels of P, Ca, and Mg [70].

Murray [71] reported that cacao leaves under shade had higher levels of nutrients than unshaded leaves. In a long-term study, Ahenkorah et al. [72] reported the beneficial effects of fertilization on cacao yield without shade. Macronutrient and micronutrient concentrations by genotype and shade level are given in Table 4. Overall macronutrient and micronutrient concentrations were at adequate levels; however, concentrations of K, Ca, and Mg were slightly higher, and P concentrations were slightly lower than the reported adequate levels in cacao [71–75].

In the current study, the interaction between cacao genotype and shade level was significant for macronutrient and micronutrient concentrations, except for N. The concentrations of all macronutrients (except for P and K) and all micronutrients were higher under 80% shade than 50% shade, and significant differences were observed between the two shade levels and across genotypes (Table 4, Tables S3 and S4). An increase of nearly 25% in the N concentration was recorded for cacao genotypes grown under the 80% shade level, contrary to the findings reported by Cabala Rosand et al. [70].

							Source of Variation [df]		
Nutrient *	Unit per Plant	PWC	PFC	BCC	NIC	Total	Shade (S) [1]	Genotype (G) [57]	G*S [57]
N 50%	$ m g~kg^{-1}$	15.09	17.45	18.26	17.20	17.01	< 0.0001	< 0.0001	0.002
N 80%	$g kg^{-1}$	19.02	21.78	22.53	21.45	21.21			
NΔ	$g kg^{-1}$	-3.93	-4.33	-4.27	-4.25	-4.20			
K 50%	$g kg^{-1}$	25.64	21.17	23.00	18.79	22.08	0.1034	< 0.0001	< 0.0001
K 80%	$g kg^{-1}$	21.82	24.66	21.52	18.53	21.63			
KΛ	$g kg^{-1}$	3.82	-3.49	1.48	0.26	0.45			
Ca 50%	$g kg^{-1}$	13.81	7.81	12.66	11.01	11.26	< 0.0001	< 0.0001	< 0.0001
Ca 80%	$g kg^{-1}$	17.47	17.67	19.08	15.14	17.31			
Ca $_{\Lambda}$	$g kg^{-1}$	-3.66	-9.86	-6.42	-4.13	-6.05			
Mg $_{50\%}$	$g kg^{-1}$	6.02	3.60	5.69	5.78	5.25	< 0.0001	< 0.0001	< 0.0001
Mg 80%	$g kg^{-1}$	6.40	7.20	7.52	5.69	6.69			
Mg $_{\Lambda}$	$g kg^{-1}$	-0.38	-3.60	-1.83	0.09	-1.44			
P 50%	$g kg^{-1}$	1.18	1.14	0.93	0.87	1.03	0.0002	< 0.0001	< 0.0001
P 80%	$g kg^{-1}$	0.74	0.80	0.54	0.83	0.73			
PΔ	$g kg^{-1}$	0.44	0.34	0.39	0.04	0.30			
Fe 50%	$mg kg^{-1}$	173.09	110.65	194.57	202.88	169.83	0.0017	< 0.0001	< 0.0001
Fe 80%	$mg kg^{-1}$	240.11	156.13	181.33	218.20	198.54			
Fe $\Delta$	$mg kg^{-1}$	-67.02	-45.48	13.24	-15.32	-28.71			
Zn 50%	$mg kg^{-1}$	40.39	32.26	27.74	39.20	34.93	< 0.0001	< 0.0001	< 0.0001
Zn 80%	$mg kg^{-1}$	47.91	46.62	44.50	43.18	45.53			
$Zn_{\Delta}$	$mg kg^{-1}$	-7.52	-14.36	-16.76	-3.98	-10.60			
Mn 50%	$mg kg^{-1}$	50.60	44.73	29.28	57.25	45.66	0.0006	0.0006	< 0.0001
Mn 80%	$mg kg^{-1}$	84.15	72.87	76.98	88.24	80.56			
Mn $\Delta$	$mg kg^{-1}$	-33.55	-28.14	-47.70	-30.99	-34.90			
Cu 50%	$mg kg^{-1}$	7.04	7.49	5.71	10.85	7.82	< 0.0001	< 0.0001	0.0017
Cu 80%	$mg kg^{-1}$	9.43	12.42	8.33	14.15	11.16			
Cu A	$mg kg^{-1}$	-2.39	-4.93	-2.62	-3.30	-3.34			

**Table 4.** Effect of shade on macro- and micronutrients concentration in 58 cacao genotypes from different origins subjected to 50% and 80% shade.

\* N: nitrogen, K: potassium, Ca: calcium, Mg: magnesium, P: phosphorous, Fe: iron, Zn: zinc, Mn: manganese, Cu: cooper. PWC: Wild cacao (from River basins of Peruvian Amazon) [n = 14], PFC: Peruvian farmers' cacao [n = 15], BCC: Brazilian cacao [n = 14], NIC: National and International cacao collections [n = 15].

Overall, cacao genotypes from the four collections showed decreases in Ca concentration when grown under 50% shade. The highest concentrations of P were 1.77 and 0.99 g kg<sup>-1</sup> for the AYP-15 (50% shade) and ICT-1281 (80% shade) genotypes, respectively (Table S3).

Slight differences were noted between cacao genotypes on leaf nitrogen concentration with respect to shade treatments, which increased with increasing shade, contrary to P, which had a higher concentration under low shade. For K concentration, decreases in shade were associated with higher leaf K; however, no significant differences were observed between the shade levels (Table 4 and Table S3).

Overall micronutrient concentrations increased significantly as shade increased 50% to 80% (Table 4). Regarding micronutrients, Fe concentration under 50% shade ranged from 72 to 346 mg kg<sup>-1</sup> for ICT-1189 and CP-53-C10, respectively, and from 120 to 389 mg kg<sup>-1</sup> for CEPEC-2002 and UF-667 at 80% shade, respectively (Table S4). The lowest concentrations for Cu were nearly 5 mg kg<sup>-1</sup> for the CP-49-C10 and UGU-112 genotypes at 50% and 80% shade, respectively (Table S4). The Zn concentration at 50% shade ranged from 21.06 to 47.89 mg kg<sup>-1</sup> for IPIRANGA-1 and UNG-77, respectively, whereas at 80% shade the concentration ranged from 36.85 to 76.39 mg kg<sup>-1</sup> for PH-21 and AYP-22, respectively. Finally, Mn concentration at 50% shade ranged from 18.42 to 100.1 mg kg<sup>-1</sup> for CA-14 and UF-667, respectively, whereas at 80% shade, it ranged from 50.23 to 155.58 mg kg<sup>-1</sup> for UNG-53 and PH-17, respectively (Table S4).

Significant differences were observed in nutrient uptake between the shade levels within each cacao collection, except for Mn (PWC, PFC, and NIC) and Cu (BCC) (Figure 2); and K, Mg, and P NUE (NIC), and Fe NUE (BCC and NIC) (Figure 3).

Under 50% shade, the highest uptakes of K, Ca, Mg, P, and Zn were found in PWC, with values of 0.61, 0.33, 0.14, and 0.03 g plant<sup>-1</sup> and 0.96 mg plant<sup>-1</sup>, respectively; whereas at 80% shade, the highest uptakes of N, K, Ca, Mg, Cu, and Zn were found in PFC, with values of 0.32; 0.37, 0.26, and 0.11 g plant<sup>-1</sup>; and 0.18 and 0.69 mg plant<sup>-1</sup>, respectively. The NIC under 50% and 80% shade showed the highest nutrient uptake values for Fe (4.73 and 2.98 mg plant<sup>-1</sup>, respectively), Mn (1.35 and 1.23 mg plant<sup>-1</sup>, respectively), and Cu (0.25 and 0.18 mg plant<sup>-1</sup>, respectively) (Figure 2).

In a field study conducted in an 8-year-old cacao plantation with different shade trees, and compared to a monoculture system, in Ghana, Isaac et al. [76] noted that nutrient uptake by cacao increased under shade (43–80% and 22–45% for N and P, respectively), and K (96–140%) was the most responsive nutrient.

Baligar et al. [43] conducted a greenhouse experiment in which cacao was grown at three shade levels: high, medium, and low shade (PPFD of  $65 \pm 25$ ,  $190 \pm 46$ , and  $1050 \pm 260 \ \mu mol \ m^{-2} \ s^{-1}$ , respectively)—combined with two levels of CO<sub>2</sub> (380 and 700  $\mu$ mol mol<sup>-1</sup>). Nutrient uptake (or content) was in the range of 158 to 168, 82 to 146, 40 to 86, 38 to 55, and 7.5 to 10 mg plant<sup>-1</sup> for N, K, Ca, Mg, and P, respectively, and in the range of 44 to 69, 410 to 538, 1127 to 1764, and 444 to 731  $\mu$ g plant<sup>-1</sup> for Cu, Fe, Mn, and Zn, respectively. At ambient CO<sub>2</sub> (380  $\mu$ mol mol<sup>-1</sup>), an increase in PPFD to 1050  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, except for K and Mn content, had a negative effect on all the essential macro- and micronutrient contents. This reduction in nutrient uptake could be attributed to a reduction in dry matter accumulation at a very high level of PPFD.

Our values for U were higher than those reported by Baligar et al. [43], and higher at 1000  $\pm$  50 (50% shade) than at 400  $\pm$  50 µmol m<sup>-2</sup> s<sup>-1</sup> (80% shade). These differences could be because, in the current study, plants were grown for a longer period (180 days vs. 57 days) and with a wide collection of cacao genotypes.

Significant differences were observed in NUE of macronutrients and micronutrients between the shade levels and within each cacao collection (Figure 3). With the exception of NUE for P in PWC, PFC, and BCC, and K in PWC and BCC, increasing shade from 50% to 80% reduced NUE for all the nutrients. However, Baligar et al. [43] reported that at ambient  $CO_2$  (380 µmol mol<sup>-1</sup>), increasing PPFD from 65 to 1050 µmol m<sup>-2</sup> s<sup>-1</sup> decreased the NUE of all macronutrients and micronutrients, with the exception of NUE for N and Ca, which increased. In a recent study, Baligar et al. [61] showed that increasing PPFD from 100 to 400 µmol m<sup>-2</sup> s<sup>-1</sup> increased uptake and NUE for all macro- and micronutrients in seven cacao genotypes.

## 3.4. Cacao Genotypes Tolerant to Shade

Shade tolerance is a complex property of plants that is achieved by different sets of responses, such as alterations in leaf physiology and biochemistry, leaf anatomy and morphology, and/or plant architecture [4]. Many methods have been proposed to measure the degree of shade tolerance of several plant species, such as sapling ratios (number of saplings growing in a low-light environment over the total abundance of the species), abundance-based index (number of stems, leaf density), mortality rate, and relative growth (which is assumed to be larger in shade-tolerant species) [1,2]. Differences observed in shade tolerance are mostly related to variations among the species' adaptations of their photosynthetic apparatus to low light intensity. The effective growth of plants at low irradiance requires the capacity to efficiently catch the available light and convert it into chemical energy, maintain a low rate of respiration, and use a large fraction of the carbohydrate pool for leaf growth [39,57]. In contrast, shade-intolerant species tend to respond to high light regimes with a significant increase in photosynthetic capacity, reduced leaf expansion, decreased branching, and early flowering. These responses are known as shade avoidance syndrome (SAS), which is one of the best-studied forms of plant phenotypic plasticity [4,77].



**Figure 2.** Nutrient uptake (U, macronutrients in g plant<sup>-1</sup> or micronutrients in mg plant<sup>-1</sup>,  $\pm$  SE) of cacao genotypes subjected to two levels of shade (50%, 80%). PWC: wild cacao (from river basins of Peruvian Amazon) [n = 14]; PFC: Peruvian farmers' cacao [n = 15]; BCC: Brazilian cacao [n = 14]; NIC: national and international cacao collections [n = 15]. All paired bar graphs that have an \* above have significant differences and those with "ns" above do not have significant differences between shade levels (Scott and Knott test,  $p \le 0.05$ ).



**Figure 3.** Effect of shade on nutrient uptake efficiency (NUE, macronutrients in g shoot  $g^{-1}$  or micronutrients in g shoot  $mg^{-1} \pm SE$ ) of cacao genotypes subjected to two levels of shade (50%, 80%). PWC: wild cacao (from river basins of Peruvian Amazon) [n = 14]; PFC: Peruvian farmers' cacao [n = 15]; BCC: Brazilian cacao [n = 14]; NIC: national and international cacao collections [n = 15]. All paired bar graphs that have an \* above have significant differences and those with "ns" above do not have significant differences between shade levels (Scott and Knott test,  $p \le 0.05$ ).



In our study, significant differences were observed across cacao genotypes regarding STI, in addition to a moderate variability within replicates. This index varied from 25.72% to 76.15% for AYP-22 (PWC) and ICS-1 (NIC) genotypes, respectively (Table S2, Figure 4).

**Figure 4.** Shade tolerance index (STI,  $\pm$ SD) of 58 cacao genotypes subjected to two levels of shade (40%, 80%). PWC: wild cacao (from river basins of Peruvian Amazon) [n = 14]; PFC: Peruvian farmers' cacao [n = 15]; BCC: Brazilian cacao [n = 14]; NIC: national and international cacao collections [n = 15]. Genotypes were classified into three groups: sensitive to shade (STI%  $\leq$  40), medium shade-tolerant (40 < STI%  $\leq$  60) and tolerant to shade (STI% > 60).

The genotypes sensitive to shade were: AYP-22, PAS-91, PAS-93, PAS-105, and UGU-112 from PWC; ICT-1026 and ICT-2653 from PFC; PH-09, IPIRANGA-1, CP-53-C10, CP-49-C10, and CEPEC-2002 from BCC; and TSH-565 an IMC-67 from NIC (Figure 4). By contrast, cacao genotypes tolerant to shade were: UGU-130 and UNG-77 from PWC; ICT-1506, ICT-2171, ICT-2142, ICT-1087, ICT-2172, and ICT-2173 from PFC; PH-144, PH-21, CA-14, and PH-990 from BCC; and POUND-12, UF-613, ICS-39, and ICS-1, from NIC. The remaining genotypes were classified as medium shade-tolerant (Figure 4).

The growth characteristics of some shade-sensitive cocoa genotypes (CP-53-C10 [BCC] and PAS-105 [PWC]), and shade-tolerant cocoa genotypes (UF-13 [ NIC] and UNG-77 [PWC] subject to 50% and 80% shade are shown in Figure 5.



**Figure 5.** Seedlings captions of growth characteristics of cacao genotypes—both sensitive and tolerant to shade. The center figure shows the greenhouse with the plant distribution, note the net that provides 50% shade (grey color) and at the end net that provides 80% shade (black color).

## 3.5. Interaction between Growth, Physiological Parameters, and Nutrient Uptake

The results of the general PCA for the growth, physiological, and nutrient uptake variables under two levels of shade with sensitive and tolerant cacao genotypes are shown in Figure 6. The first two PCA axes explained 54.5% of the overall variation of genotypes under 50% shade (Figure 6A) and 69.2% of the overall variability of genotypes under 80% shade (Figure 6B).

Under 50% shade, the first PCA axis accounted for 31.6% of the overall variation and was related to negative values with high values of S/R, and positive values with high values of CHL, Ca, and Mg, which were positively correlated among themselves and negatively correlated with SL, SD, RDW, and Cu; the sensitive genotypes PAS-105, CP-53-C10, PH-09, and CP-49-C10 were related to Ca, Mg, and Fe uptake. Positive values related to genotypes with high values of BDW, SD, RDW, WUE, N, K, Mn, P, and Cu, which were also positively correlated among themselves. Proportions of 71.4% and 37.5% of sensitive and tolerant genotypes, respectively, were related to macro- and micronutrients, CHL, and WUE. The second principal component, which explained 22.9% of the overall variance, was represented mainly by variations in negative values of growth parameters (RDW, SD, SL, LA, RA) and Cu uptake, and by variations in positive values of the remainder of the nutrient and physiological parameters. The axis divided positively 71.4% of sensitive genotypes, and 50% of tolerant genotypes.



**Figure 6.** PCA analysis of shade effect on growth, physiological parameters, and nutrient uptake of shade-sensitive and tolerant genotypes, subjected to 50% shade (**A**) and 80% shade (**B**).

Under 80% shade, the first PCA axis accounted for 56.5% of the overall variation and was related to negative values of CHL and S/R, and divided 100% and 12.5% of sensitive and tolerant genotypes, respectively. In positive values, we found that all shade-tolerant genotypes were related to physiological parameters and nutrient uptake.

Based on these results, we can infer that the sensitive and tolerant genotypes have better development at 50% shade, whereas at 80% shade the tolerant genotypes are directly related to nutrient uptake, physiology, and growth parameters. This characteristic of shadetolerant genotypes permits better development of plants and selection of genotypes that withstand the high level of shade, whereas the sensitive genotypes do not have a positive relationship with the nutrient uptake, physiology, and growth parameters.

Shade intolerant species usually exhibit greater physiological plasticity, which allows them to achieve rapid growth rates, probably associated with more effective net assimilation rates than with structural traits [39,78] The increase in light intensity when photosynthesis and growth rate are faster causes a decrease in total N in the leaf (which is the source of proteins, chlorophyll, etc.) and a decrease in P. This implies that P uptake cannot keep up with increased growth at higher light levels [57].

In cacao genotypes sensitive to shade, it is expected that growth parameters have a strong correlation with almost all nutrients, which is the case of RDW in tolerant genotypes. By comparison, only shade-sensitive cacao genotypes showed a positive correlation between RDW and N content in the aboveground tissues. This is probably because a long and branched root system is necessary to increase water and nutrient capture [79], which may be reduced under heavy shade conditions.

Under controlled conditions, some plants grown hydroponically have shown a decline in chlorophyll content as the Mn concentration increased [80,81], whereas in other cases chlorophyll content decreased as the Mn content decreased [82]. In the case of cacao, all genotypes showed a negative but not significant correlation between these two parameters.

Finally, shade-sensitive and tolerant cacao genotypes exhibited negative correlations between Cu and chlorophyll content. Several authors observed that increasing levels of Cu in the nutrient solution or in soil were associated with a decrease in stomatal conductance, which causes a decline in photosynthetic gas exchange [83–85], or that an increase in Cu lowered the leaf chlorophyll concentration, enhancing sensitivity to photoinhibition [86].

# 4. Conclusions

The quality and quantity of PPFD that reaches the canopy of cacao trees leaves grown in field conditions are very different than PPFD that reaches the canopy of cacao leaves under artificial shade. In field-grown cacao upper story tree leaves filter out most of the light so the red: far-red ratio changes depending on the greater or lesser absorbance and/or transmittance of electromagnetic light. Therefore, the cacao genotypes' responses to two levels of shade (PPFD) in this research are based on response to artificial shade

Fifty-eight cacao genotypes grown under greenhouse conditions and subjected to 50% shade (PPFD of  $1000 \pm 50 \ \mu mol \ m^{-2} \ s^{-1}$ ) and 80% shade (PPFD  $400 \pm 50 \ \mu mol \ m^{-2} \ s^{-1}$ ) were evaluated to determine their growth, physiological, and nutrient-use-efficiency responses, and tolerance to shade. For almost all growth, physiological, and nutritional parameters, interactions between shade levels and genotypes were statistically significant; therefore, it was not possible to establish significant differences for each factor individually. Only maximum root length, leaf area, N concentration, Cu content, and Cu efficiency appeared to vary significantly between shade levels and across genotypes.

Overall results suggest that heavy shade negatively affects cacao, except for shoot length, leaf and root area, shoot/root ratio, and chlorophyll content, which were usually higher at 80% than at 50% shade.

From the wild cacao, Peruvian farmers (or ICT), Brazilian, and national and international cacao collections, only 28% of the cacao genotypes evaluated were identified as being tolerant to shade, whereas 21% were sensitive to shade.

Total dry weight and WUE showed a strong relationship with almost all macronutrient and micronutrient contents in cacao plants that were tolerant to shade. In addition, these plants also had a higher total dry biomass than the sensitive genotypes.

The cacaos with shade-tolerant were, from PWC: UNG-77 and UGU-130; from PFC: ICT-1506, ICT-2171, ICT-2142, ICT-1087, ICT-2173; from BCC: PH-144, CA-14, PH-21 and PH-990; from NIC: POUND-12, ICS-39, ICS-1 and UF-613.

The results of this study indicate that cacao genotypes adapted to unfavorable conditions, such as high shade, could be used in breeding programs as a strategy to breed shade-tolerant cacao cultivars, thereby maintaining sustainable cacao production under agroforestry systems.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10 .3390/agronomy11081536/s1, Table S1: Growth parameters (mean values per plant) of cacao genotypes from different origins subjected to two levels of shade (50% and 80%); Table S2: Physiological parameters (mean values)- and Shade Tolerance Index (STI) for 58 cacao genotypes subjected to two levels of shade (50 and 80%); Table S3: Mean concentrations of macronutrients (g kg<sup>-1</sup>) in shoots of 58 cacao genotypes subjected to two levels of shade (50 and 80%); Table S4: Mean concentration of micronutrients (mg kg<sup>-1</sup>) in shoots of 58 cacao genotypes subjected to two levels of shade (50 and 80%).

**Author Contributions:** E.A.-G. implemented the original idea, planned the experimental details, wrote the manuscript, carried out the research and data analysis. A.F. collected and compiled the reported data. F.B. took the lead in writing the manuscript, organized and interpreted the data, and conducted the statistical analysis. C.O.A.-H. performed the plant analysis and worked out the correlation matrix of all the observed parameters. L.B.Z.-C. assisted with the implementation and data analysis. J.A. assisted with planning and implementation of the experiment. V.C.B. conceived the study and was in charge of the overall direction and planning. All authors provided critical feedback during the writing process. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Data presented in this study are available on request from the corresponding author.

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