COFFEE: FROM THE FIELD TO THE CUP

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COFFEE: FROM THE FIELD TO THE CUP

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Table of Contents

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Editorial: Coffee: From the field to the cup

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Editorial on the Research Topic
Coffee: From the Field to the Cup

Along with the pages of this volume, the reader will meet many more challenges than solutions, which we hope will motivate new studies and development of innovative approaches to improve coffee cultivation under the looming challenges of climate change. Since the discovery of use of coffee as a beverage and its expansion of cultivation to regions outside of Ethiopia and Yemen, coffee cultivation has not been without challenges.

Catarino et al. enlightens how atmospheric elevated CO_2 concentrations and progressive drought affect the coffee-plant physiology and metabolism. Young coffee plants were grown under actual and enriched CO_2 (800 ppm) concentrations and either exposed or not exposed to water deficit. Photosynthesis and water use efficiency was improved even under drought. There were changes in the biochemical composition of leaves, markedly a decrease in phenolic compounds under elevated CO_2, regardless of the water regime. Chlorogenic acid and caffeine increased under the combination of water deficit and elevated CO_2.

Nitrogen (N) is the most abundant nutrient taken from the soil by plants, and nitrate (NO_3) is the main nitrogen form absorbed by plants, which transport through the root cell membranes is mediated by low- and high-affinity membrane transporters. Bohórquez et al. studied the variations in N uptake during four coffee fruit developmental stages. Rapid fruit expansion was the developmental period when the kinetic parameter V_{Max} showed a positive relationship with bean production. They also observed that at maturation, because of a decrease in carbohydrate demand, fine roots were more abundant than at the grain filling stage. Because these fine roots mostly absorb nutrients, water shortage might compromise N uptake because of root mortality.

The prospection and maintenance of coffee germplasm is crucial to breeding coffee programs aiming to produce plants adapted to the global warming scenario. In this regard, two articles were published on this Research Topic. Krishnan et al.

used simple sequence repeat (SSR) markers to assess the genetic diversity of wild and cultivated populations of Arabica coffee from the Boma Plateau in South Sudan, which has been suggested as a center of origin of *Coffea arabica* species, in addition to Ethiopia. Comparing these populations with accessions from Ethiopia, Yemen, and global cultivars, they confirmed their hypothesis and showed that the wild population analyzed was genetically distinct from Ethiopian Arabica. Because Arabica coffee is an autogamous species, thus with low genetic variability, South Sudan coffee population can be a rich material to be explored in breeding programs.

In another paper, Dullo et al. evaluated the genetic accessions found in the coffee germplasm of CATIE International Coffee Collection (CICC) in Turrialba, Costa Rica, which has genotypes from all over the world, including from missions organized by several organizations to collected wild genetic material in Ethiopia. The methodological approach they created for an in-depth assessment of the collection can be used for other collections and help breeding programs.

To face the challenge of climate change and ensure resilience to coffee plantations, Davis et al. consider the relocation of the coffee plantations to more suitable areas and replacing the available cultivars with new hybrids and/or species as appropriate strategies. Aiming to improve our knowledge of two coffee species, *C. racemosa* and *C. zanguebariae*, and based on the lacunas of several previous works, they studied the phylogenetic and spatio-phylogenetic relationship to confirm or refute their existence as separate species. They also studied their climate requirements and agro-climatic suitability, obtained preliminary sensory (flavor) information, and provided primary agronomic data. The authors concluded that both species have traits that could be explored in breeding programs. Among the traits, the authors cited heat tolerance, low precipitation requirement, high precipitation seasonality (dry season tolerance) and rapid fruit development, which are characteristics considering global warming and changes in the water regime in traditional areas of coffee plantations.

Because of the narrow genetic basis and low diversity of *C. arabica*, Medeiros et al. integrated molecular characterization, genetic diversity analyses, and circulating diallel studies aiming to develop a strategy to select new cultivars. They used molecular markers to assess the genetic diversity of 76 candidate parents and verify the crossing of potential F1 hybrids. They selected eight elite parents for circulating diallel analysis. The parents and 12 hybrids were evaluated based on 10 morpho-agronomic traits. Medeiros et al. concluded that with this approach it is possible to identify elite plants which can be used in breeding programs to develop new cultivars in response to global climate changes.

Coffee quality is a result of the genetic background, environment and fruit processing, and the interactions among these three factors. Malta et al. studied the importance of the genetic background in the beverage quality of 31 elite cultivars of *C. arabica*. The fruits were wet-processed, and the sensory profile of the beans was characterized and compared using multivariate statistical tools. The authors could discriminate the genealogical groups using chemometric analysis, Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA). Using this approach, they selected three elite cultivars with scores equal to or above 90 points. The authors discuss the influence of the genetic background of each group in the results obtained.

C. arabica originally grew in the understory forest of Ethiopia, but nowadays, cultivars of this species are primarily grown in full sunlight. In recent years several crops have been cultivated under agroforestry systems. The coffee agroforestry system has been adopted in many tropical countries as a sustainable practice bringing better and more stable income, mainly for smallholder farmers. Bertrand et al. proposed breeding coffee to select plants productive under shade conditions. They replicated an experiment in the shade and sun and, following several characteristics, reached conclusions regarding the selection of candidate varieties suitable for the coffee agroforestry system, which could target different markets. For example, by choosing the F1 hybrids, they believe it is possible to increase productivity under shade and full sun and at the same time select for good sensory qualities.

Shading has also been adopted in coffee plantations in the Central Highlands, the main coffee-growing area of Vietnam. Le et al. analyzed the transition of sun cultivation coffee to shade in a small ethnic minority village in Lâm Đ*ông province. They observed improvement in the biodiversity and that the soil of the shade-grown coffee farms was enriched with organic matter. Because of a decrease in the inputs, they also found that the net return in the shade system was almost four times higher than in the full-sun system.

Siles et al. also studied the process of small farmers' adoption of coffee agroforest systems in Nicaragua. The authors found several gaps still to be filled regarding food and income benefits. Decreasing coffee margins, labor scarcity, pests, and climate variability allows small coffee farmers changes regarding coffee varieties and associated trees. The authors also had important insights to improve data collection, enabling a more in-depth analysis to design new strategies for agroforestry transformation in Nicaragua.

Venzon reviewed the adoption of an agroforestry system, cover crops, and non-crop plant management in coffee plantations as sustainable tools to control coffee pests. Fruit and wood trees used to generate biodiversity could provide an additional income for small coffee farmers. Additionally, the production of coffee under these systems may support small farmers entering the specialty coffee market.

Koutouleas et al. comprehensively reviewed the literature on coffee shading as a strategy to grow coffee and mitigate and adapt the crop to future climate changes. The review gives elements to the reader plan and anticipate strategies for future challenging climate conditions, as well as provides elements for future research, from breeding programs to practical management of the coffee-agroforestry system.

Ripper et al. were the first to compare the composition of the exotics coffees Jacu and Kopi Luwak and report a comprehensive chemical analysis. The data showed some consistency regarding changes in the chemical composition of both exotic coffee, mainly caffeine and chlorogenic acid.

Author contributions

HPM and PM wrote the first draft of the editorial. SK, SALA, and MBAG made corrections to the text. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Kinetic Parameters of Nitrate Absorption by Adult Coffee Trees

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Nitrogen, the most demanded nutrient by coffee plants, has a rate of recovery from the soil of about 50%. Because of that high doses of nitrogenous fertilizers are used to reach high production, and consequently high amounts of N are lost to the environment. Knowing the kinetic parameters of nitrate (NO_3^-) absorption over the fruit development cycle is important as a mean of achieve more adjusted fertilizer doses and better recovery rates for the N applied as fertilizers. This study aimed determining the kinetic parameters of NO_3^- absorption in different development stages of fruits from adult coffee plants. The kinetic parameters V_{max} and K_m were determined in a low production year, at the pinhead (PH), rapid expansion (RE), grain filling (GF), and maturation (MT) stages. One month before each kinetics assay, lateral roots of eight plants were excavated and wrapped into non-woven fabrics grow cylinders filled in with vermiculite to produce absorbent roots. On the assay day, the roots were washed and immersed into a container with 1 L of 90 μmol L^{-1} NO_3^- solution. Sampling began one and a half hours after that, and was taken every hour over 7 h. Data on NO_3^- depletion were used to calculate the absorption kinetic parameters V_{max} and K_m. In a low production year the V_{max} ranged from 0.14 to 0.72 μmol g^{-1} h^{-1} in a root fresh matter basis and K_m from 6.47 to 50.31 μmol L^{-1}. The V_{max} values were highest at the PH and MT stages; the lowest absorption rate was recorded at GF and K_m was lowest at RE. As at the RE stage of fruits V_{max} shows a positive correlation with grain production, adequate nitrogen availability must be ensured before this phase to not to affect coming coffee production.

Keywords: V_{max}, K_m, phenological stages, absorption rate, nitrogen

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INTRODUCTION

Nitrogen (N) is an important nutrient for plant growth and development. This nutrient participates of several components of the photosynthetic apparatus and enzymes involved in CO_2 fixation, determining biomass production, and thereby is considered an important factor in crop productivity. Among the available forms of N, nitrate is absorbed in greatest amounts by plants. NO_3^- uptake can range from 0.1 to 5.0 mmol L^{-1} in cultivated soils (Bose and Srivastava, 2001). Nitrate (NO_3^-) is also the N form most absorbed by coffee roots (Miller et al., 2007; Reis et al., 2007). However, due to losses in the soil by volatilization and leaching, the recovery efficiency of nitrogen fertilizers is low, between 40 and 50% of the dose applied. The loss of N applied as urea (three top dressings a year) was reported to be 31.2% in coffee orchards located at the Lavras region, in Brazil (Dominghetti et al., 2016). In addition to the economic

impact, these losses cause negative environmental impact due to gaseous N volatilization and contamination of waterways by leached NO_3^-.

Absorption mechanisms enable plants to adjust to environmental fluctuations in the concentrations of nutrients and their different ionic forms. In the case of N, transport proteins in the plasma membranes, which have different affinities for both nitrate and ammonium, mediate the absorption of nitrate and ammonium by roots. Plants developed a refined absorption system for nitrate with high-affinity transport system (HATS) that operate in low external concentrations (<0.5 mmol L^{-1}) of the ion, whereas low-affinity system operate in high concentrations (>0.5 mmol L^{-1}), thus ensuring the absorption of nitrate according to the ion availability in the substrate (Marschner, 2012; Wang et al., 2012).

Difficulties in quantifying N fractions in the soil have limited the nutrient recommendation based on estimates of this availability by chemical analysis and hindered reliable predictions of their effective availability to plants. Therefore, kinetic parameters of N uptake may provide information on the ability of plants to acquire NO_3^- at a given concentration in the substrate. Kinetic parameters also allow the plant to serve as a sensitive indicator of the fertilizer required to meet the nutritional demands, which can increase the agronomic efficiency of nitrogen fertilizers. In this sense, recent works have measured the kinetic absorption parameters as a mean of selecting rootstocks of peach, grapevine and pear more efficient in the absorption of nitrate-nitrogen and ammonium-nitrogen (Paula et al., 2018; Kulmann et al., 2020; Sete et al., 2020). Relatively to coffee-plant the estimated kinetic parameters of NO_3^- uptake in 9.5 month-old plants of eight coffee varieties grown in growth chambers were in average 3.47 μmol g^{-1} h^{-1} for V_{max} and 236.3 μmol L^{-1} for K_m. The varieties showed significant differences among them. "Catuaí Amarelo" presented high V_{max} and low K_m without or with water stress, meanwhile Mundo Novo although presenting low V_{max} and low K_m did not suffer alterations in these parameters when under water stress (Martinez et al., 2020).

Kinetic parameters have been traditionally estimated using hydroponic systems because of the ease of collecting root samples and depletion solution without major methodological complexities. However, few references are found on studies conducted with adult plants, and in field conditions the lack of information is even greater, despite these estimates could be more realistic.

Another important consideration is that root morphological characteristics like diameter, length, surface and volume showed to be highly related to kinetic parameters of N absorption (Kulmann et al., 2020), and these root characteristics are known for changing during coffee berry development (Magalhães, 2021).

When cultivated at full sun *Coffea arabica* presents biennial production cycle as a result of abundant flowering and of the drain force of developing grains (Cannell, 1985). In a high fruit production year the load of fruits impairs the vegetative growth of productive branches, in which the fruiting would occur in the following year, but this reduced vegetative growth is preceded by death of thin absorbent roots (Rena and Maestri, 1987). This way, a relationship between production and root growth parameters is expected. In fact Silva et al. (2020) verified positive correlation between *Coffea canephora* production and root length, surface and volume.

Considering these features of adult coffee plants, as an approach of the field conditions Pinto (2016) used a drip system to estimate the kinetic parameters of NO_3^- absorption at different phenological phases of coffee fruit development using 5-year-old trees of variety Catuaí Vermelho IAC 99 cultivated in vases containing sand, in a greenhouse. The author found that K_m was highest at the stage of the first rapid expansion, intermediate at the pre-flowering and flowering-to-pinhead, and lowest at grain filling. V_{max} was lowest at grain filling compared with the other stages.

The objective of this study was to estimate the kinetic parameters of NO_3^- absorption in 5 years old coffee plants at different phenological phases of fruit development, using methodology developed for field conditions.

MATERIALS AND METHODS

Experimental Conditions

The study was carried out in the coffee experimental plantation of the Federal University of Viçosa (20° 45' 58" S latitude; 42° 52' 06" W; 676 m altitude). The soil of the area is classified as Red-Yellow Latosol (Jacomine, 2009). The climate of the region is of the type Cwa, according to the Köppen classification, hot and humid with dry cold winter and minimum temperatures below 10°C. **Figure 1** shows the temperature and relative air humidity variations over the experimental period.

Five-year-old coffee trees of cultivar Catuaí Vermelho IAC 99, in the low production year of the biennial cycle, formed the stand in this study. The coffee was planted at the spacing 2 × 1 m in the rainfed system. The crop has been maintained with usual management practices, liming and fertilization according to Ribeiro et al. (1999), since planting. During the experimental phase, the plants were kept free from crop-weed competition through mechanical weeding. Pest and disease management was carried out according to the technical recommendations for coffee cultivation.

The experimental plot comprised 10 rows with 75 plants each, totaling 1,500 m^2. **Table 1** shows the results of the soil chemical analysis at the beginning of the experiment. The analysis was done according to the methods described by Defelipo and Ribeiro (1997) and Alvarez et al. (2000).

Before the beginning of the crop season, in August 2017, liming was carried out with 1.65 t/ha of dolomitic limestone (CaO 30% and MgO 10%) with 77% TRNP, according to Ribeiro et al. (1999). Fertilizers were applied at the outside edge of the crown by applying 100, 35, and 45 g/plant of ammonium sulfate, simple superphosphate and potassium chloride in early November, late December 2017 and, mid-February 2018. One cycle of irrigation was applied immediately after fertilization.

Nitrate Depletion Assays

At the end of the previous crop cycle, in July 2017, 32 trees were randomly selected for homogeneity in size, number of

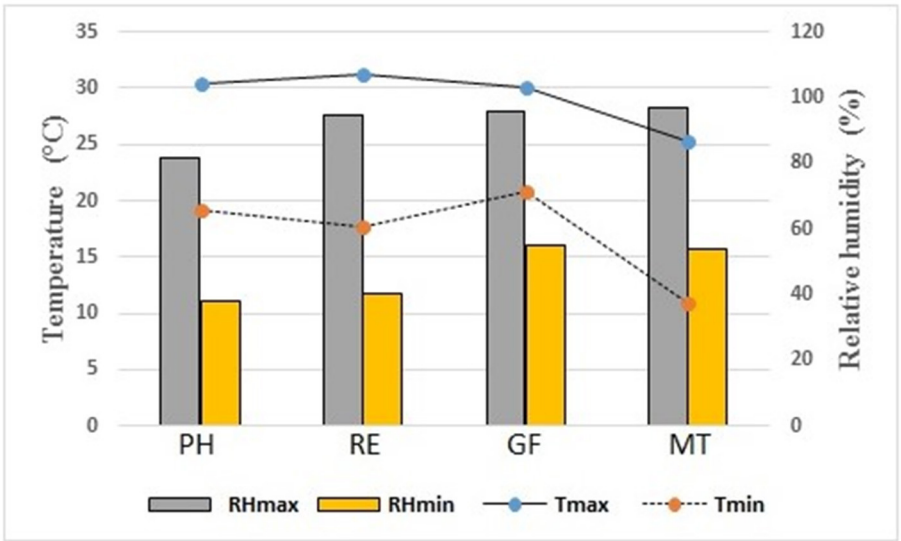

FIGURE 1 | Daily relative humidity (RH), and daily temperature (T) over the absorption kinetic assays.

TABLE 1 | Soil chemical characteristics at the beginning of the coffee reproduction cycle.

Chemical characteristics	0–20 cm depth
pH[a]	4.44
P (mg/dm^3)[b]	3.1
K (mg/dm^3)[b]	102
Ca (cmol$_c$/dm^3)[c]	1.35
Mg (cmol$_c$/dm^3)[c]	0.27
Al (cmol$_c$/dm^3)[c]	2.30
H + Al (cmol$_c$/dm^3)[d]	17.2
t (cmol$_c$/dm^3)	4.18
T (cmol$_c$/dm^3)	19.08
V (%)	9.9
m (%)	55
Cu (mg/dm^3)[b]	16.09
Fe (mg/dm^3)[b]	484.3
Mn (mg/dm^3)[b]	3.1
Zn (mg/dm^3)[b]	3.30
P rem (mg/L)[e]	19.8
Organic matter (dag/kg)[f]	1.57

[a]pH in water, 1:2.5. [b]P, K, Zn, Mn, Fe, and Cu: Mehlich-1 extractant. [c]Ca^{2+}, Mg^{2+}, and Al^{3+}: KCl 1 mol L^{-1} extractant. [d]H + Al: Ca(OAc)$_2$ 0.5 mol L^{-1} pH 7.0 extractant. [e]P-rem: Remaining phosphorus (Alvarez et al., 2000). [f]Organic matter: C. org. x 1.724 (Walkley-Black).

plagiotropic branches, leaves, and vigor. These plants were labeled to be used in four depletion assays that were conducted in the fruit phenological development stages pinhead (PH), rapid expansion (RE), grain filling (GF), and maturity (MT). Approximately 1 month before each sampling, 8 trees among the 32 selected were picked at random. Lateral roots near the trunk, in the 0–10 cm soil layer under the crown, were exposed by careful digging trenches (50 cm long, 20 cm wide and 20 cm deep). When the lateral root was reached, it was wrapped into a NWF (non-woven fabric), forming a root growth chamber (8 cm diameter and 30 cm long) filled with vermiculite.

The root growth chamber consisted of a 15 × 30 cm NWF rectangle placed under the root selected, which was surrounded by vermiculite, then wrapped in the shape of a cylinder, and tied in place with plastic string. The distal end of the root was cut at the base of the cylinder, while the proximal end remained attached to the plant. The trench was filled with the excavated soil surrounding the cylinder to support the root. The growth chamber remained below the soil surface until the depletion assays were performed. Afterwards, the soil surrounding the growth chamber was removed to expose the newly developed absorbent roots. The roots were placed into a plastic tray, washed with deionized water, and placed into urine-like collection bags filled with 1 L of the depletion solution attached to a portable aerator. The bags had an aperture on the top to introduce the roots. The apparatus was isolated from the underlying soil by polyethylene film to avoid contamination of the depletion solution (**Figure 2**).

The depletion solution contained 90 μmol L^{-1} of NO_3^- in the form of KNO_3. Absorption took 90 min to reach the steady state. Then, every hour, about 2 mL of the depletion solution were collected through the drain tap at the bottom of the bag, for 7 h (between 9:30 am and 4:30 pm). The samples were transferred into a previously weighed Eppendorf tube and quickly placed in a box with ice. At the end of the process, the final volume of the depletion solution was measured to determine the volume lost by transpiration. Fine roots (Ø ≤ 2.00 mm) were cut from the selected lateral root, washed, separated from the thick roots, and weighed in an analytical balance to determine fresh matter.

The main flowering of the coffee plants occurred on 10/9/2017 and the samplings were carried out on 11/16/17, 12/10/17,

FIGURE 2 | Exhaustion assay description: **(A)** Excavation, lateral permanent root identification, and packaging in substrate; **(B,C)** Withdrawal of substrate and washing of fine roots; **(D)** Packing of fine roots in a bag containing exhaustion solution; **(E,F)** Exhaustion solution sampling.

03/14/18, and 5/11/18 at the PH, RE, GF, and MT stages, according to the duration of the phenological phases determined by Laviola et al. (2009) in this region. We choose sunny days to perform the four assays. The total radiation over the canopy during each assay was of 18,587.70; 22,032.00; 15,919.10, and 13,169.98 kJ/m^2, respectively.

In the laboratory, the Eppendorf tubes were re-weighed to determine the exact volume of each sample and the nitrate concentrations in the aliquots were measured. The nitrate concentrations in the depletion solution were determined by the colorimetric method as described by Doane and Horwath (2003), using a Biochrom EZ 400 READ molecular absorption spectrophotometer, in the 540 nm wavelength.

Kinetic Parameters Determination

The volumes of depletion solution available to the plant at the sampling times were calculated using an electronic spreadsheet, taking into account the amounts removed by the samplings and the transpiration estimated. The water loss by transpiration was considered constant over the sampling period of 7 h.

The methodology of Claassen and Barber (1974) was used to calculate the kinetic parameters of absorption. The method relates the amount of the nutrient (obtained by multiplying the concentration by the volume of the depletion solution available to the plant at each sampling) to the sampling time. For each plant assayed, a function nutrient quantity × time was obtained, which was the basis for estimating the kinetic parameters. The mathematical-graphical method described by Ruiz (1985) was used to calculate V_{max} and K_m. The method divides the aforementioned function into a linear segment for calculating V_{max} and another asymptotic segment for calculating K_m. V_{max} was estimated considering the fresh matter weight of absorbent roots, expressed as $\mu mol\ g^{-1}\ h^{-1}$.

The values obtained for V_{max}, K_m were inserted in to the Michaelis -Menten equation to provide better explanation of the nitrate absorption process in RE, GF, and MT fruit development phases:

$$v_0 = V_{max} \frac{C}{K_m + C}$$

Where:

$v_{0=}$ instantaneous velocity of NO_3^- absorption;
V_{max} = maximum NO_3^- absorption velocity;
C = NO_3^- external concentration;
K_m= NO_3^- concentration at which ½ V_{max} is reached.

Fine Root Production per Plant and Bean Production per Plant

The production of absorbent roots by each plant was estimated at the GF and MT stages. For the amount of absorbent roots per tree, soil samples of known volume were collected from the 0 to 30 cm layer with a 7.5 cm diameter soil probe. Samples were collected from three sampling points inside the canopy projection area of each assayed tree. The samples were removed from the probe, placed in trays, and roots were separated, washed and weighed. The weight of roots with diameter bellow 2 mm present in the soil volume sampled was then extrapolated to the volume of the cylinder estimated by the average crown diameter to the 30 cm depth, in which about 90% of the absorbent roots of the coffee tree are concentrated (Inforzato and Junqueira,

1963, Motta et al., 2006; Ronchi et al., 2015; Silva et al., 2020; Magalhães, 2021).

After complete maturity, coffee cherries of each assayed plant were picked and weighed to determine their fresh matter weight.

Statistical Analysis

Although we have had assayed 32 plants, eight in each phenological stage of fruit development, not all results fit to a expected biological trend for nitrate absorption. For the exhaustion assays we had suitable results to two plants in the PH stage, four plants to the RE and MT stages and five plants to the GF stage. The kinetic parameter data were presented as descriptive statistics (mean ± standard deviation). In addition, Pearson's correlation coefficients were calculated between the kinetic parameters V_{max}, K_m, fresh weigh of roots, and fruit production.

RESULTS

Kinetic Parameters of Nitrate Absorption

The V_{max} kinetic parameter varied between 0.143 and 0.724 μmol g^{-1} h^{-1} and the K_m varied between 6.476 and 50.316 μmol L^{-1}. Both of them varied according to the phenological phases of fruit development (**Table 2**). The mean confidence interval at the maturity stage shows that V_{max} was significantly higher to those at the rapid expansion and bean filling stages; however, it was statistically similar to the result observed at the pinhead stage.

It was not possible to determine K_m for the pinhead stage in the depletion assays performed, because in this phase the depletion adjusted to a linear model. The values of K_m for the other stages increased with the year round and the succession of the phases of fruit development. The lowest values were achieved at the rapid expansion stage and the highest values at maturation stage (**Table 2**).

The Michaelis -Menten equation applied to these data, showed that nitrate uptake exhibited saturation kinetics, as expected for the range of low external concentrations (**Figure 3**). The absorption rate up to the concentration of 20 μmol L^{-1} was higher at the RE stage than at the stages of GF and MT and was achieved with the lowest nitrate external concentrations due to its lowest K_m value. Above 20 μmol L^{-1}, the absorption rate was the highest at the maturation stage due to its highest V_{max} value, despite its highest K_m. At the GF stage, the nitrate absorption rate was the lowest among the stages evaluated, which was influenced to a greater extent by the lowest V_{max} (**Figure 3**).

Correlations Between Kinetic Parameters, Production of Fine Roots and Beans

In this study, the root system fresh matter of plants was evaluated at the GF and MT stages. The GF stage was selected because it shows great competition between fruits and vegetative organs for photoassimilates (Valarini et al., 2005; Laviola et al., 2006). Maturity was selected because of the lowering of the photoassimilates demand by fruits in this stage, allowing the recovery of root growth. At the GF stage, the plants presented, on average, four times less fresh matter of fine roots than in the MT stage (**Figure 4**).

TABLE 2 | Nitrate absorption kinetic parameters: maximum absorption velocity (V_{max}) and Michaelis-Menten constant (K_m) in four coffee-plant phenological phases.

Phenological phases	V_{max} μmol g^{-1} h^{-1}	K_m μmol L^{-1}
Pinhead	0.493 ± 0.372	–
Rapid expansion	0.268 ± 0.134	6.476 ± 1.003
Grain filling	0.143 ± 0.037	15.285 ± 1.517
Maturity	0.724 ± 0.183	50.316 ± 3.874

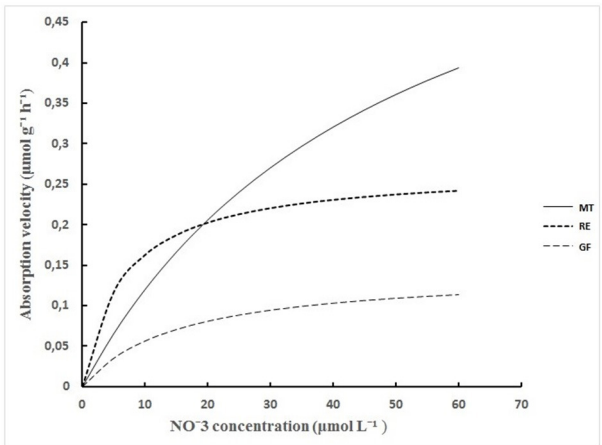

FIGURE 3 | Nitrate absorption velocity by adult coffee trees (μmol g^{-1} h^{-1}) as a variable of its concentration in the external solution (μmol L^{-1}) in three phenological phases of fruit development: RE (rapid expansion), GF (grain filing), and MT (maturation).

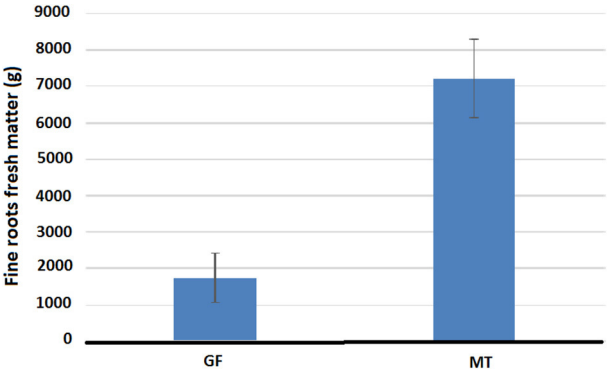

FIGURE 4 | Fresh matter of fine roots of 5-year old coffee-trees in the phenological phase of grain filing and maturation of fruits.

The average cherry production was 10.51 t/ha, corresponding to 1.65 t/ha of coffee beans with moisture content of 11%. It is of note that coffee has a biennial production cycle and that during the study the crop was in a year of low production and without irrigation, which lead to relatively low average yields.

TABLE 3 | Pearson's correlation coefficients between fruit production, root fresh matter and kinetic parameters.

Rapid expansion			Grain filing			Maturation		
	V_{max}	Prod		V_{max}	Prod		V_{max}	Prod
V_{max}	1		V_{max}	1		V_{max}	1	
Prod	0.95	1	Prod	−0.34	1	Prod	−0.32	1
K_m		0.16	RFM	−0.57	0.57	RFM	−0.28	−0.42
						Km		−0.14

The fruit production of the trees (Prod) used in the depletion assays was correlated with the NO_3^- absorption velocity during the fruit rapid expansion stage (0.95 Pearson correlation coefficient). On the other hand, the V_{max} of NO_3^- absorption obtained at the GF and MT showed a weak negative correlation with production. V_{max} decreased at these later stages, suggesting a slightly lower NO_3^- uptake capacity of the most productive plants at bean filling. The correlation between V_{max} and fresh root mass was also negative. A positive correlation, although not very high (0.57), was found between the fresh matter of fine roots at grain filling and production. V_{max}, at maturity, had a negative correlation with low degree of association with Prod. V_{max} at the grain filling stage had a negative correlation with root fresh matter. In contrast to the response at the GF stage, root fresh matter at the MT stage had a negative correlation with Prod (**Table 3**).

DISCUSSION

Kinetic Parameters of Nitrate Absorption

The range of values of the kinetic parameters observed in field conditions, between 0.143 and 0.724 $\mu mol\ g^{-1}\ h^{-1}$ for V_{max} and between 6.476 and 50.316 $\mu mol\ L^{-1}$ for K_m (**Table 2**) agrees with the results obtained by Pinto (2016) in greenhouse conditions. This author have worked with 60-month-old coffee plants of the same variety under no water stress, in a drip hydroponic system with sand substrate, and found values of V_{max}, ranging between 0.34 and 0.83 $\mu mol\ g^{-1}\ h^{-1}$. For the K_m, this author found values between 120.48 and 264.98 $\mu mol\ L^{-1}$, which were considerably higher than the results of the present work. These differences are likely to be due to different experimental conditions, especially the nitrate concentration in the depletion solution, the fresh matter of fine roots, and the environmental conditions.

The confidence interval of the mean at the maturity stage (**Table 2**) shows that V_{max} was significantly higher in this phase than those at the rapid expansion and bean filling stages; however, it was statistically similar to the result observed at the pinhead stage. Epstein and Bloom (2006) state that V_{max} is defined by the multiplication of the number of absorption sites (transporter proteins) per root unit and its operation velocity, whereas Li et al. (2013) state that V_{max} refers to the maximum capacity of the root system to absorb a particular nutrient when all transporters are saturated, indicating maximum transport rate. It was seen, therefore, contrary to what was expected based on the demands of the fruit and contrary to expectations that the maximum capacity of nitrate uptake occurs at the pinhead and maturity stages, either by increasing the number of the transporters or by increasing its operation velocity.

In agreement to our results Neto et al. (2014) found that the NO_3^- leaf content increased from the pre-anthesis to the pinhead stage with a subsequent drop at the grain filling stage. This drop is attributed to N remobilization to support fruit growth. However, the authors observed an increase in leaf NO_3^- levels at the beginning of fruit ripening and high Nitrate Reductase activity at pre-anthesis. Similarly, Carelli et al. (1989) found high Nitrate Reductase activity at pre-anthesis and at the end of fruit development, indicating the intense metabolism and demand for N at these stages. Carvajal et al. (1969) and Reis et al. (2009) also found in mature coffee plants higher levels of nitrate absorption at pre-anthesis and at the beginning of fruit ripening.

Our observation of the highest V_{max} values at the pinhead and maturity stages is quite consistent with the foliar concentrations and patterns of the Nitrate reductase activity found in the studies mentioned in the previous paragraph. This suggests that V_{max} responded to the nitrogen demands at these stages leading to greater or lower nitrate uptake and that the mechanisms operating the absorption manage to modulate this parameter as a function of the different nutrient demands signalized by the phenological phases. Additionally, Laviola et al. (2006) observed a reduction in the leaf N content of three varieties of *Coffea arabica* between the phases of flowering and suspended growth, followed by a small recovery in this phase and later decline during the grain-filling/maturity. Prezotti and Bragança (2013), on the other hand, found that nitrogen contents in both the leaf and the root of *Coffea canephora* decreased with time after flowering, indicating occurrence of nitrogen remobilization during fruit development and suggesting a greatest uptake in the phases that precede those with the greatest demand. Covre et al. (2018) studied the accumulation of macronutrients along the development of fruits of *Coffea canephora* and the concomitant variation in their contents in the leaves adjacent to them. These authors also verified a recover of leaf N content in the maturation phase in both, irrigated and non-irrigated plants.

Fernandes and Sousa (2006) mention that the control of nitrogen uptake has been related to the nitrogen status in the plant, showing that high levels of reduced forms of nitrogen (NO_2^-, NH_4^+ and free amino acids) lead to reduced uptake, *via* feedback control, indicating that the levels of reduced compounds in the plant are related to the nitrate absorption dynamics. Ivashikina and Sokolov (1997) found that the supply of reduced nitrogen compounds (ammonium and the amino acid glutamine) decreased the activity of nitrate reductase and increased the K_m values for nitrate. Thus, the status of reduced nitrogen compounds in the plant can contribute to the regulation of the nitrate uptake kinetic parameters.

Unfortunately it was not possible to determine K_m for the pinhead stage because, in this case, the depletion of NO_3^- over the time adjusted to a linear model. It is worth to note that in this early stage of fruit development the raining season was only beginning, after a long period of dry season. Because of that, few new fine roots had developed in the lateral roots selected and wrapped into vermiculite cylinders. The values of K_m for

the other stages increased with the successive phases of fruit development, with the lowest values been verified at the rapid expansion stage and the highest values at maturation stage. K_m is defined as the solution concentration at which half of V_{max} is reached and indicates the affinity of the transporters for a particular form of the nutrient, thus the lower the K_m values, the greater the transporters' affinity with the ion and the greater its transport toward the interior of the root cells. These results show that the greatest transporter's affinity for nitrate occurred at the rapid expansion stage, but there was a reduction at the grain filling stage and even a greater reduction at the maturity stage. In view of that we can hypothesize that the highest V_{max} at pinhead and bean filling is caused by the greater number of transporters at these stages, since its turnover was lower, as indicate by the K_m values observed.

The Michaelis-Menten equation was used for a better interpretation of our data. The equation is adapted to the kinetics of nutrient uptake, and describes the absorption rate of a solute as a function of its external concentration considering V_{max} and K_m. The nitrate uptake by plants exhibited saturation kinetics as expected, in the range of low external concentrations (**Figure 3**). The absorption rate up to the concentration of 20 μmol L^{-1} was higher at the RE stage than at the stages of GF and MT and was achieved with the lowest concentrations due to its lowest K_m value. This indicates that the nitrate uptake was more efficient in low concentrations. Above 20 μmol L^{-1}, the absorption rate was the highest at the maturation stage due to its highest V_{max} value, despite its highest K_m, indicating that, in this case, probably a greater number of transporters contributed to a greater absorption, compensating for the lower affinity of the transporter for nitrate.

At the GF stage, the nitrate uptake rate was the lowest among the stages evaluated, which was influenced to a greater extent by the lowest V_{max}, indicating that at this stage the number of carriers or their operating velocity was low (**Figure 3**). In agreement to these results, Pinto (2016) working with coffee plants of the same variety and age cultivated in hydroponic sand culture also verified the lowest V_{max} at GF stage.

Correlations Between Kinetic Parameters, Production of Fine Roots and Beans

In addition to the kinetic parameters, nutrient absorption also depends on the morphological characteristics of the roots, length, diameter, surface area, and their distribution in the soil profile. A greater root system length indicates a greater volume of soil explored by the roots, with greater potential for nutrient absorption, in which the surface area and length are the most relevant characteristics for nutrient uptake efficiency (Zonta et al., 2006).

In this study, the root system fresh matter of plants was evaluated at the GF and MT stages. The GF stage was selected because it shows great competition between fruits and vegetative organs for photoassimilates (Valarini et al., 2005; Laviola et al., 2006). At the GF stage, the maximum daily rates of carbohydrate accumulation, especially starch, are achieved in the fruits (Laviola et al., 2007), which sometimes, according to plant production, results in great level of death of absorbent roots. Maturity stage was selected because of the low carbohydrate demand by fruits, which can allow the recovery of root growth.

At the GF stage, the plants presented, on average, four times less fresh matter of fine roots than in the MT stage, which could be associated with root death caused by the high demand of the fruits for carbohydrates and nutrients due to its strong sink activity (**Figure 4**). Mengel and Barber (1974) suggested that fruit development may affect root growth, since after pollination, in corn, root density in the topsoil layer decreased. Nutman (1933) reported that a heavy fruit load in the coffee tree severely reduced the supply of carbohydrates to the roots leading to their death. The greater amount of fine roots at the maturity stage (near the end of the rainy summer time) may indicate the recovery of root growth, which contributes to meet the nutrient demands at this stage, or otherwise, to give support to accumulate reserves for the growth of the next production cycle. In this regard, DaMatta et al. (1999) found that the nitrogen supply during the dry and cold autumn-winter, after harvest, in which the coffee plants stop growing, provided an increase in the nitrogen concentration and the activity of Nitrate Reductase in the leaf in the next active growth phase, which begins with flowering in the spring. The authors found that the nitrogen compounds accumulated in the root were reallocated to shoots, which could support higher growth rates. Therefore, these functional relationships would depend on a greater amount of fine absorbent roots at the maturity stage, which together with a higher V_{max}, would increase the reserves of nitrogen compounds for further use in the new crop cycle.

In the same orchard used in this study, Magalhães (2021) studied the variation in root length, surface, volume and fresh matter over the phenological cycle of coffee fruit production during 2 years, one of low production and the other of high production. He observed that in the year of high production length, area, volume and fresh matter of thin roots (with <2 mm diameter) located in the first 20 cm soil layer decreased from rapid expansion up to maturation, while in the year of low production the reverse occurred. Such finding shows that the previous year production will determine in some extent the amount or the growth rate of roots available for nutrient absorption in the beginning of the subsequent cycle. This evidences that in addition to the kinetic parameters we need considering the fluctuations that occur in the amount of absorbent roots throughout the stages of fruit development in order to modeling properly nitrogen fertilization doses at each stage of fruit development and year of the biennial cycle of production. Anyway, the results obtained in this study corroborate the observation of Laviola et al. (2006) that the availability of N must be adequate already in the phase of rapid expansion of the fruits, and the first N fertilization need to be done before that.

The average cherry production was 10.51 t/ha, corresponding to 1.65 t/ha of coffee beans with moisture content of 11%. It is of note that the experiment was done in a year of low production and without irrigation, which leads to reduction in the average yields.

The fruit production of each tree (Prod) was correlated with the V_{max} of NO_3^- uptake during the rapid expansion stage (0.95 Pearson correlation coefficient, **Table 3**). According to Laviola et al. (2009), in this same region, the RE stage has the highest daily rates of DM and N accumulation in the fruits. This indicates that at the RE stage there was accumulation of nitrogen or nitrogen compounds in the plant, which afterwards, via reallocation, contributed to complete the fruit growth.

On the other hand, the V_{max} of NO_3^- uptake obtained at the GF and MT showed a weak negative correlation with production (**Table 3**). V_{max} decreased at these stages, suggesting a slightly lower NO_3^- uptake capacity of the most productive plants at bean filling, which would indicate a negative feedback of N uptake in plants that had high uptake at the rapid expansion stage. The correlation between V_{max} and fresh root mass was also negative, indicating a possible compensatory effect of increased uptake velocity as the amount of roots decreases, which seems consistent in the sense of maintaining a stable absorption rate in the face of a decrease in acquisition tissue. Edwards and Barber (1976) observed that decreasing the proportion of corn roots exposed to nitrogen-containing nutrient solution increased N uptake rate per root unit, but not enough to compensate the deleterious effect of the root length reduction.

A positive correlation, although not very high (0.57), was found between the fresh matter of fine roots at grain filling and production. This may indicate that a larger amount of roots at this stage would have little influence on fruit production, which corroborates the hypothesis that the N uptake during the rapid expansion meets the demand during the later fruit growth. In fact, V_{max} at this stage was numerically lower than at the other stages.

The nitrate V_{max}, at maturation, had a negative correlation with low degree of association with Prod (**Table 3**) despite showing the highest value among the studied phenological phases, indicating that, as expected, at this stage nitrate V_{max} has little influence on the current coffee production. Similar to what occurred at the GF stage, at the MT phase nitrate V_{max} had a negative correlation with root fresh matter, possibly indicating that increase in the amount of roots has a regulatory effect on nitrate V_{max} as already mentioned. In contrast to the response at the GF stage, root fresh matter at the MT stage had a negative correlation with Prod, which reinforces that, at this stage, a larger amount of fine roots apt to absorb nitrate has no influence on the current fruit production.

CONCLUSIONS

Coffee-trees nitrate absorption rates are dependent on the phenological phases of fruit development, showing that the absorption mechanisms can modulate the kinetic parameters according to the different demands at each fruit development stage.

In low productivity years the coffee-tree nitrate V_{max} ranges from 0.14 to 0.72 $\mu mol\ g^{-1}\ h^{-1}$ in a root fresh matter basis and the K_m from 6.47 to 50.31 $\mu mol\ L^{-1}$.

In low productivity years the nitrate V_{max} of coffee-tree is highest at the pinhead and maturation phenological phases and lowest at the grain filling phenological phase. The lowest K_m occurs in the rapid fruit expansion.

At the rapid expansion stage of fruits V_{max} shows a positive correlation with grain production, so before this stage, adequate nitrogen availability in the soil must be ensured so as not to affect coming coffee production.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

CA has planned and performed the experiment, he has also done the statistical analysis, discussed the data and wrote the first draft of the paper. HM has planned and advised the experimental activities, discussed the results and wrote the final version of the paper. RS has helped in planning the experiment and discussing the results. All authors contributed to the article and approved the submitted version.

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Selection of Elite Genotypes of *Coffee arabica* L. to Produce Specialty Coffees

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This study aimed to evaluate the cup quality of *Coffea arabica* elite genotypes submitted to wet processing. *C. arabica* elite genotypes, which were grouped according to their genealogy: Bourbon, Paraíso Germplasm, and Resistant to Rust. Coffees were sent to wet processing to obtain fully washed coffee. After processing and drying the coffees were subjected to cup quality analysis according to the methodology of the Association of Special Coffees (SCA). To characterize and discriminate the genealogical groups the data were submitted to chemometric analysis, Principal Component Method (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA). The PCA was effective in presenting an overview of the data, demonstrating the variables that most contributed to the analysis response. However, the PCA was not efficient to group genotypes according to their genealogical origin, based on chemometric data, as it is an unsupervised analysis. Even though most of the samples were classified correctly, the PLS-DA model created has not yet managed to correctly classify the genotypes of the Paraíso germplasm group. The *C. arabica* elite genotypes evaluated have the potential to produce special coffees, especially on the genotypes Paraíso 2, H493-1-2-10 and UFV-7158 with scores equal to or above 90 points.

Keywords: coffee breeding, coffee quality, post-harvest, multivariate analysis, chemometric analysis

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INTRODUCTION

The consumption in the world of specialty coffees has been growing in much greater proportions than the consumption of ordinary coffees, mainly due to changes in the behavior of consumers who seek not only its stimulating effect, but also pleasure and satisfaction when tasting the coffee beverage (Fassio et al., 2019a).

The beverage quality of the coffee is dependent to the intrinsic quality of the coffee beans (Figueiredo et al., 2013), which is expressed by the chemical composition originated from the interaction between genotype x environment x processing (Borém et al., 2016; Fassio et al., 2019a; Malta et al., 2020). These compounds will, after roasting, develop the characteristic aroma and flavor of the beverage, in addition to other important sensory attributes such as body, acidity and sweetness (Figueiredo et al., 2015; Borém et al., 2016).

The genetic component has an important influence on the determination of flavor and aroma of coffee. *C. arabica* species is the one with the greatest potential to produce coffee of superior

quality within the *Coffea* genus (Teressa et al., 2010; Tessema et al., 2011; Fassio et al., 2019a; Malta et al., 2020). *C. arabica* is originally from Ethiopia and has been propagated and disseminated worldwide from a small number of plants, which has led to a narrow genetic basis within Arabica coffee cultivars (Scholz et al., 2016). To increase genetic variability, several introductions have been made since 1928 and transferred to germplasm banks around the world, used to produce new, more productive cultivars, adapted to growing regions and resistant to diseases (Kathurima et al., 2009; Tessema et al., 2011; Gimase et al., 2014; Scholz et al., 2016). Within this context, several elite progenies are in the final selection phase and will soon be able to constitute new genetic materials available for cultivation.

Due to the importance of the genetic component as well as the form of processing used to determine the cup quality, the purpose of this investigation was to evaluate the quality potential of 31 elite genotypes of *C. arabica* from Epamig's improvement program submitted to wet processing and to characterize the sensory profile of the elite genotypes, using multivariate statistical tools in view of the use of a model created by the PLS-DA method to classify and discriminate Arabica coffee genealogical groups Bourbon (GB), Paraiso Germplasm (GP) and Rust-resistant (GR) according to the sensory profile of these genotypes.

TABLE 1 | Coding and identification of the *C. arabica* L. elite genotypes evaluated.

Genealogical group	Genotype code	Genotype identification
Bourbon Group (GB)	G3	Red Bourbon (MG 0035)
	G4	Yellow Bourbon (MG 0009)
	G6	Red Bourbon (MG 0011)
	G7	Orange Bourbon (MG 0025)
	G10	Yellow Bourbon (MG0073)
	G13	Red Bourbon (MG 0064)
	G15	Yellow Bourbon (MG 0049)
	G16	Yellow Bourbon (MG 0026)
	G17	Yellow Bourbon (MG 0020)
	G18	Yellow Bourbon (MG 0052)
	G21	Yellow Bourbon (MG0036)
	G26	Red Bourbon (MG 0014)
Paraíso Germplasm (GP)	G8	MGS Paraíso 2
	G23	Paraíso MG H419-1
	G24	H419-6-2-5-2
	G33	H419-10-6-2-10-1
	G36	H419-3-3-7-16-4-1
	G39	H419-10-6-2-12-1
	G40	H419-10-6-2-5-1
Resistant Group (GR)	G2	Oeiras
	G9	Sarchimor MG 8840
	G11	Obatã Vermelho IAC 1669-20
	G20	H493-1-2-10
	G25	UFV 7158
	G28	Catiguá MG3
	G29	Araponga MG1
	G30	Catiguá MG1
	G31	Catiguá MG2
	G32	Sacramento MG1
	G35	Acauã
	G37	Pau-Brasil MG1

MATERIALS AND METHODS

Sampling, Experiment Location, and Processing

The experiment was carried out in the 2015 and 2016 agricultural years with coffee samples from 31 elite genotypes of *C. arabica* L. from Epamig's improvement program and partner institutions which were separated into groups according to the genealogical origin (**Table 1**). The three genealogical groups are formed by genotypes that have stood out in relation to the cup quality according to evaluations where protocols of the Association of Special Coffees—SCA were used.

The elite genotypes evaluated in this study are implemented in the field, in a randomized block design with two replications and 10 plants per plot, in the Empresa de Pesquisa Agropecuária de Minas Gerais—EPAMIG (Agricultural Research Company), located at 18°59'26" south latitude, 48°58'95" west longitude, and 975 meters altitude, in the Alto Paranaíba region.

The coffee fruits were collected when most were at the ideal maturation point and processed by the wet method to obtain fully washed coffee (**Figure 1**). The grains were dried in screened bottom sieves and periodically revolved until reaching a water content of 11% (w.b.). After drying, the coffee samples were packed in a Kraft® paper bag and covered with a polyethylene bag. The samples were stored in a cold chamber at ± 18° C for a period of 30 days.

Sensory Analysis

The sensory analysis of the coffee was carried out according on the protocols described by the Specialty Coffee Association—SCA (Lingle, 2011). One hundred grams of coffee beans were roasted from until they reached the color standard # 65 for whole grains of the Agtron/SCA Color Classification System. For cup quality, a panel of three trained judges (Q-grader) evaluated five cups of each coffee sample in relation to ten sensory attributes: fragrance/aroma, uniformity, absence of defects, sweetness, flavor, acidity, body, balance, completion and overall impression. The final sensory score was generated from the sum of the evaluated attributes, with coffees with a score equal to or >80 points being considered special coffee.

Data Analysis

The sensory evaluation data for the 31 elite genotypes of *C. arabica*, were analyzed using multivariate statistical tools such as the principal component method (PCA) and partial least squares discriminant analysis (PLS-DA) using the statistical software R (R Development Core Team, 2017).

PCA is an unsupervised multivariate method that aims to find new variables that are not correlated in such a way as to explain the maximum variation of the original data set (X) without referring to any class label (Y). These new variables are the principal components and it is desirable that they be

correlated with the original variables, in order to reduce the original dimension of the variables in a smaller dimension of variables, constituted by the principal components (Malta et al., 2020). In this sense, the PCA was used for the purpose of evaluating the similarity between the sensory attributes, formed by matrix X, in the different genotype codes, as mentioned in Sampling, Experiment Location, and Processing.

The partial least squares discriminant analysis (PLS-DA) was used to separate the genotypes in the different genealogical groups. PLS-DA is a supervised method that uses the desired response (Y) to build a model that classifies a sample considering the variables in matrix X and its respective category (Y) for a given group (Fassio et al., 2019b). The method consists of modeling the structure of variance and covariance of latent variables in such a way as to maximize the multidimensional variance of the variables in matrix X in the direction of matrix Y. It should be noted that, due to the existence of a correlation between the sensory attributes, the usual linear regression methods, and conventional discriminant analysis (Linear Discriminant Analysis-LDA) would not be adequate (Taveira et al., 2014).

The PLS-DA model was developed to separate the genealogical groups of elite genotypes of *C. arabica* submitted to wet processing according to sensory attributes. To assess the model's performance, the classification error rate for each component of the PLS-DA was considered (Rohart et al., 2017). The load of each variable in the component was also used, which provided in a better error rate to indicate the variables in matrix X that presented the greatest contributions to the PLS-DA component.

RESULTS

Multivariate Analysis

In the multivariate analysis, only the sensory attributes of acidity, body, flavor, and aroma were evaluated, together with the final sensory score, as the other attributes didn't show differences in the scores for evaluated genotypes.

Principal Component Analysis (PCA)

PCA was used as a first approach to multivariate analysis to obtain an overview of the data. **Figure 2** is a projection of the results obtained in the PCA, regarding the distribution of genotypes according to the sensory attributes analyzed and the final sensory score. The first two principal components explained 97.91% of the response variability (95.88% for PC1 and 2.03% for PC2). The Final Score variable presented the highest loading value for PC1 and, therefore, the greatest influence (0.9981) in the separation of the evaluated genotypes. However, all sensory attributes were important in the formation of PC1. The variables Body and Flavor were determinants for the formation of PC2 (**Table 2**).

It was observed that the genotypes grouped in the rightmost region of the biplot present greater intensity of the attributes of acidity, flavor, fragrance, and final sensory score (**Figure 2**). Once the PC1 was determined by all sensory attributes and final score, all of them presenting positive coefficients (**Table 2**), it is noted that the coffees that are located in the bars in the positive direction of the graph (**Figure 3A**) are the ones that present

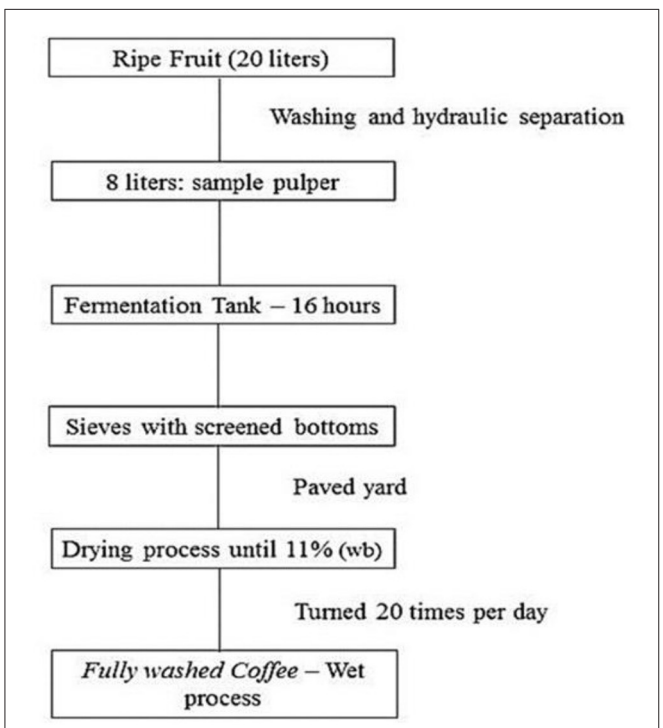

FIGURE 1 | Flowchart of the wet processing method adopted to obtain fully washed coffee.

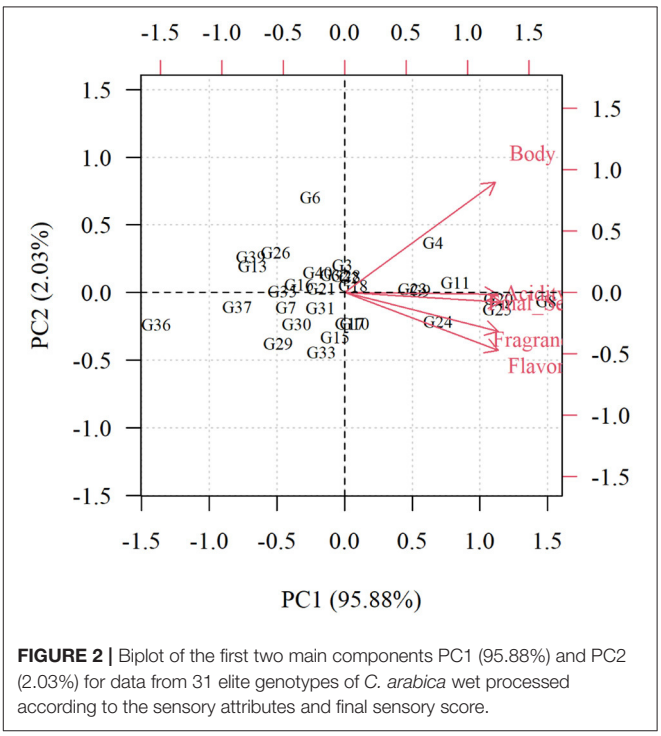

FIGURE 2 | Biplot of the first two main components PC1 (95.88%) and PC2 (2.03%) for data from 31 elite genotypes of *C. arabica* wet processed according to the sensory attributes and final sensory score.

TABLE 2 | Correlations between the evaluated parameters (final score and sensory attributes) with the first two main components and the respective coefficients of each parameter with the main component.

Parameters	PC1 (95.88%)		PC2 (2.03%)	
	Coefficients	Correlations	Coefficients	Correlations
Fragrance	0.4474	0.9796	−0.2961	−0.0943
Flavor	0.4474	0.9795	−0.4428	−0.1410
Acidity	0.4462	0.9770	−0.0142	−0.0045
Body	0.4391	0.9613	0.8430	0.2684
Final Score	0.4559	0.9981	−0.0729	−0.0232

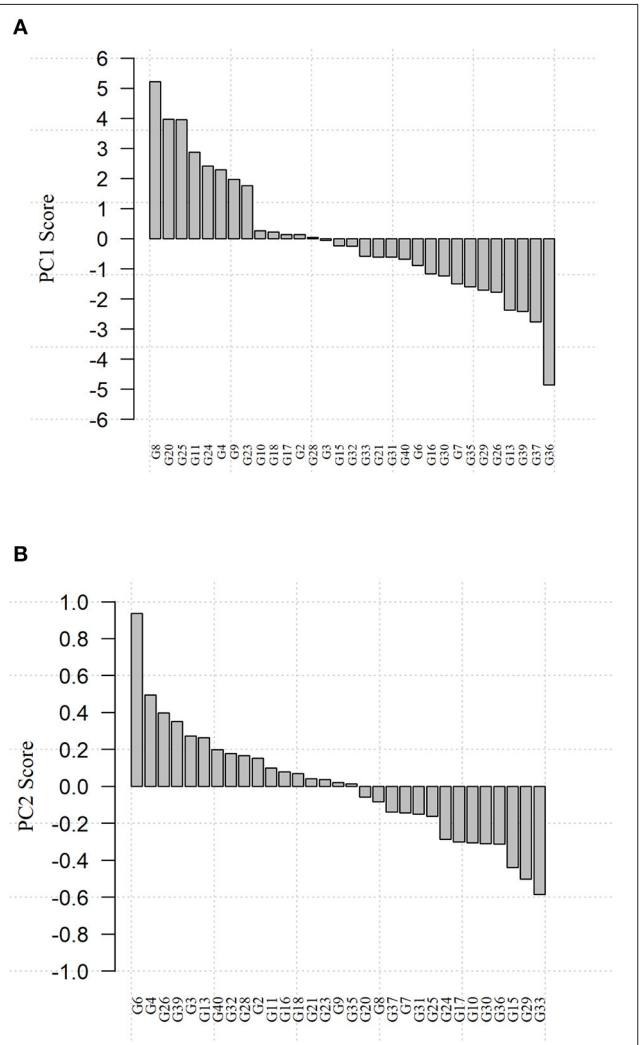

FIGURE 3 | Scores of PC1 **(A)** and PC2 **(B)** for data from 31 *Coffea arabica* elite genotypes processed by wet process according to the scores of sensory attributes and the final score.

highest values of all sensory attributes and final score, with emphasis on the G8, G20, and G25 genotypes. The G8 genotype (MGS Paraíso 2) belongs to the Paraíso germplasm. The G20 genotype (H493-1-2-10) comes from a cross between the cultivar Red Catuaí IAC 144 with Timor Hybrid. The G25 genotype (UFV 7158) belongs to the Catimor germplasm and comes from the crossing between Red Caturra with Timor Hybrid. It should be noted that all the highlighted genotypes have in their ancestry the Timor Hybrid germplasm, which shows that it is possible to obtain specialty coffees when using this germplasm as a source of resistance to rust. On the other hand, coffees with bars in the negative direction of the graph are those with the lowest values of sensory attributes, with emphasis on the genotype G36 (H419-3-3-7-16-4-1), which belongs to the Paraíso Germplasm.

The second principal component (PC2) can be understood as a comparison between the sensory attributes of flavor and body, that is, low values of the first imply in high values of the second, according to the adjusted coefficients of the PC2 (**Table 2**). Thus, according to PC2, the G6 genotypes (Red Bourbon) and G4 (Yellow Bourbon) have higher scores for the body attribute and lower scores for the flavor attribute. In contrast, the G33 genotype (H419-10-6-2-10-1) has higher scores for the flavor attribute and lower scores for the body attribute (**Figure 3B**).

Applying the PLS-DA Model

Through the principal component analysis, it was possible to establish a relationship between the genealogical groups and the sensory attributes and the final score. However, such an analysis does not quantify the relationship of a specific sensory attribute with that group. In this sense, PLS-DA analysis was used.

The scores obtained by the PLS-DA show the similarity between the genotype codes (**Figure 4A**), so that most of the genealogical groups are included in the ellipses of 95% confidence. It is observed that there are genealogical groups that are overlapped by the confidence ellipses of other groups, which can make it difficult to discriminate by the model. Supposedly, the GP group has similar characteristics to the GB and GR groups, which justifies the high error rate in the classification of genotype codes in the genealogic groups (**Table 3**).

Figures 4B,C (graph of the correlation circle and variable loads in component 2 of the PLS-DA) show the variables that most influenced the classification of genotypes in the groups. The attributes body, acidity, and final sensory score further influenced the classification of the group of cultivars resistant to rust (GR).

Through cross-validation, the performance of the PLS-DA model was assessed, returning an overall error rate of 56.71% (**Table 3**). The genotype codes data set were divided into five

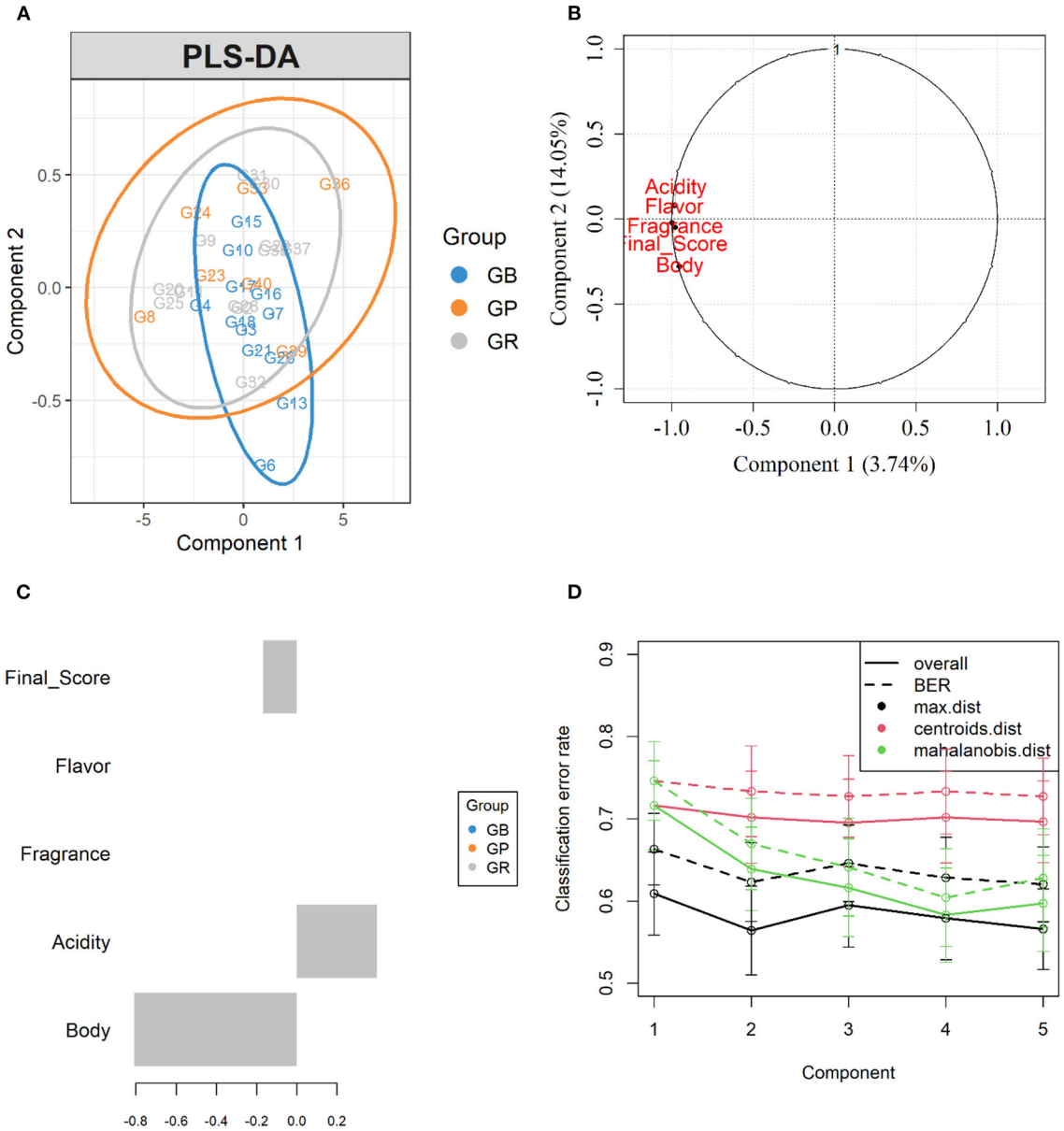

FIGURE 4 | (A) Graph of the PLS-DA scores of two components for sensory analysis differentiating the genealogical groups. (●) GB—Bourbon Group, (●) GP—Paraíso Germplasm, (●) GR—Resistant Cultivars Group. **(B)** Correlation Graph of sensory attributes with components 1 and 2 with classes GB, GP and GR. **(C)** Loads of component 2 of the PLS-DA model of sensory attributes for each genealogical group. **(D)** Classification error rate generated for the components of the PLS-DA model through a cross-validation procedure using five parts (5-fold) of the data set to adjust the model and the rest to test it in 100 simulations.

parts through 100 Monte Carlo simulations and one part was used to obtain the error rates. In this sense due to the similarity of the GP genealogical group with the other groups, the model did not correctly classify these samples and most of them were classified as belonging to the resistant group (GR). The ideal number of components in the model is 2 (**Figure 4D**) and the sensory attribute Body has the greatest contribution to the classification of genealogical groups, followed by Acidity (**Figure 4D**).

In each group, it is possible to identify the highlighted genotypes for these variables, such as the G20 and G25 genotypes for the rust resistant group (GR) and the G6 and G13 genotypes for the Bourbon group (GB), properly classified by the PLS-DA, according to the **Figures 4A**, **5**.

According to the results presented in **Table 3**, it appears that two samples with genotype code from the GP were classified by the PLS-DA model as being from the GB genealogical group and five samples as being from the GR group. Therefore, of the seven

TABLE 3 | Counting false positives and negatives in reference to the classification of genealogical groups by the PLS-DA model and their respective error rates obtained through a cross-validation procedure using 5 parts (5-fold) of the data set to adjust the model and the rest to test the same in 100 simulations.

Genealogical Groupobserved	Genealogical Group classified by PLS-DA model			TOTAL
	GB*	GP*	GR*	
GB*	8	0	4	12
GP*	2	0	5	7
GR*	4	0	8	12
TOTAL	14	0	17	31
Classification error rate	33.33%	99.29%	55.25%	-
Global error rate	56.71%	-	-	-

**GB: Bourbon Group; GP: Paraíso Germplasm; GR: Resistant Group.*

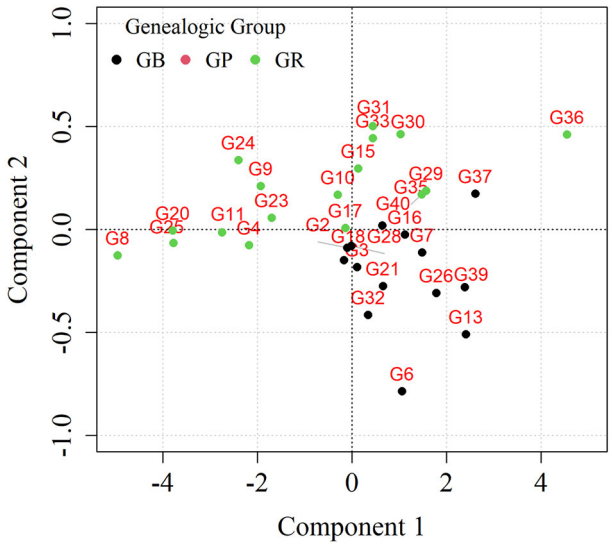

FIGURE 5 | Graph of the PLS-DA scores of two components for the sensory attributes with the classifications of the genealogical groups. (●) GB-Bourbon Group, (●) GR—Resistant Group (●) GP—Paraíso Germplasm.

total samples in the GP group, none were classified correctly. Then, the diagonal formed by the numbers 8, 0, 8 are the correct classifications of the PLS-DA model created.

Even though most of the samples were classified correctly, the model created still failed to correctly classify the genotypes of the Paraíso Germplasm (GP) group. It is important to point out that the accesses belonging to the Paraíso Germplasm group come from crossings between Yellow Catuaí and Timor Hybrid. Therefore, it appears that just like the group of cultivars resistant to rust (GR), the Paraíso germplasm group presents in its ancestry the germplasm Timor Hybrid, which may have made it difficult to separate these two groups.

DISCUSSION

In this investigation, two multivariate analysis were employed to evaluate and discriminate elite genotypes, processed by the wet method (fully washed), for the sensory analysis of coffees, the PCA method and the PLS-DA method. These methods were applied together for the separation of *C. arabica* genealogical groups regarding the quality of the coffee beverage.

The PCA method identified the behavior of most of the genotypes with respect to analyzed variables. It is known that the chemical compounds of the raw coffee beans are important precursors of the aroma and flavor of roasted coffee (Fassio et al., 2019b). In addition, genetic variability influences both the chemical composition and the physical properties of coffee beans, and these, in turn, directly affects the cup quality (Scholz et al., 2013; Borém et al., 2016). In that regard, studying the trends of coffee genotypes in terms of beverage quality becomes essential for the development of cultivars with the most potential to produce specialty coffees (Fassio et al., 2019b).

The PCA was efficient in demonstrating an overview of the data, identifying the variables that most contributed to the analysis response. However, the PCA was not efficient to group genotypes according to their genealogical origin, based on chemometric data, as it is an unsupervised analysis. In a similar work, Malta et al. (2020), also observed that the PCA didn't presented detailed information about the differences between the group's accessions. Thus, PLS-DA method was requested as a useful tool for this proposal.

Through the PCA scores, it was possible to identify patterns between genotypes codes in relation to sensory attributes and, as reported by Borém et al. (2016) and Scholz et al. (2013), the chemical compounds from raw coffee beans are important precursors of other compounds formed in the roasting process that are relevant to the cup quality.

Since PCA is an unsupervised procedure, which does not imply the prior knowledge of genealogical groups, the PLS-DA procedure becomes a useful tool when one is interested in incorporating genealogical groups in the analysis. **Figures 4**, **5**; **Table 3** corroborate the efficiency of the method and similar discussions are raised by Malta et al. (2020).

The PLS-DA created model managed to correctly classify most genotypes of the Bourbon groups (GB) and Resistant group (GR). The GB was the one with the best classification by the PLS-DA created model; what denotes the stability of this germplasm to produce specialty coffees (Fassio et al., 2019b). It appears that although Bourbon cultivars are recognized for

their potential for producing specialty coffees, there is variability within the GB to produce superior quality coffees, with genotypes having greater potential for this characteristic than others. The Bourbon cultivar is traditionally known for the production of specialty coffees, especially in conditions of high altitude and low temperature (Ferreira et al., 2012; Figueiredo et al., 2013; Borém et al., 2016). However, it is possible to observe that under the same edaphoclimatic conditions, other materials have the same potential, or higher, than Bourbon cultivars to produce specialty coffees, which can be used in breeding programs aimed at obtaining new cultivars with high quality potential.

Within the Bourbon group there are two important variations, Red Bourbon and Yellow Bourbon. Red Bourbon was introduced in Brazil because it is more productive than the Typica cultivar and is of good quality (Ferreira et al., 2012). The xanthocarpa form (yellow fruits) has its most probable origin by the mutation of Red Bourbon or by the recombination of the natural crossing between Red Bourbon and Botucatu Yellow (Carvalho, 2008). The Bourbon cultivar is recognized worldwide for its high potential in producing specialty coffees, for its sweet taste and characteristic aroma. It is relevant to observe that the good quality of new, more modern cultivars is often attributed, in part, to the very genetic makeup of the Bourbon cultivar, as it enters directly or indirectly into the genetic makeup of new cultivars (Malta et al., 2014).

Most of the improved, rust-resistant cultivars currently in cultivation have as their source of resistance the germplasm called the Timor Hybrid, selected by the Coffee Rust Research Center (CIFC), Oeiras-Portugal. The Timor Hybrid has its origin, possibly, in a natural cross between *C. arabica* x *C. canephora* (Setotaw et al., 2010). Due to its characteristics of similarity with the cultivars of *C. arabica* and, mainly, for its resistance to rust, the Timor Hybrid has always been seen as very promising for the improvement of coffee and, therefore, it is widely used to obtain rust-resistant coffee populations, such as Catimor, Sarchimor, Cavimor, Cachimor, Blumor, and others. Several investigations corroborate that the Timor Hybrid can also be used as a source of genes to improve other relevant characteristics, such as the coffee beverage quality (Carvalho, 2008; Setotaw et al., 2010; Sobreira et al., 2015; Fassio et al., 2019a; Malta et al., 2020).

Even though most of the samples were classified correctly, the model created has not yet managed to correctly classify the genotypes of the Paraíso germplasm group (GP). The accessions belonging to the Paraíso germplasm group come from crossings between Yellow Catuai and Timor Hybrid (Malta et al., 2014). Therefore, it appears that just like the group of cultivars resistant to rust (GR), the Paraíso germplasm group has in its ancestry the Timor Hybrid germplasm, which may have made it difficult to separate these two groups (Sobreira et al., 2015). The Paraíso Germplasm gave rise to the cultivars Paraíso MG H419-1 and more recently MGS Paraíso 2, recently launched by Epamig. The cultivar MGS Paraíso 2 has a high percentage of flat grains in the highest sieves, having, on average, four harvests, 67.9% in sieves 16 and above. The high quality of beverage in this cultivar has also aroused interest in coffee growers who are dedicated to the production of specialty coffees. The aroma is sweet, with red fruit flavor, pleasant acidity, velvety body, with great sweetness (Malta et al., 2014).

CONCLUSION

The PCA was effective in presenting an overview of the data, demonstrating the variables that most contributed to the analysis response. However, the PCA was not efficient to group genotypes according to their genealogical origin, based on chemometric data, as it is an unsupervised analysis. Even though most of the samples were classified correctly, the PLS-DA model created has not yet managed to correctly classify the genotypes of the Paraíso germplasm group (GP). The *C. arabica* L. elite genotypes evaluated have the potential to produce specialty coffees, especially on the genotypes Paraíso 2, H493-1-2-10 and UFV-7158, which presented final scores equal to or >90 points.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MM, AO, and GC contributed to conception and design of the study. AP, AS, LA, and GC organized the database. GL performed the statistical analysis. MM, AO, and GC wrote the first draft of the manuscript. MM, LA, AS, and DM wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Elevated [CO_2] Mitigates Drought Effects and Increases Leaf 5-O-Caffeoylquinic Acid and Caffeine Concentrations During the Early Growth of *Coffea Arabica* Plants

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Increasing atmospheric [CO_2] is thought to contribute to changes in precipitation patterns, increasing heatwaves and severe drought scenarios. However, how the combination of elevated [CO_2] and progressive drought affect plant metabolism is poorly understood. Aiming to investigate the effects of this environmental condition on photosynthesis and specialized metabolites in leaves of *Coffea arabica* during the early growth, plants fertilized with ambient (a[CO_2]-400 ppm) and elevated (e[CO_2]-800 ppm) [CO_2] were exposed to well-watered (WW) or water-deficit (WD) regimes for 40 days. Over the 40-day-water-withdrawal, soil moisture, and leaf water potential decreased compared to WW-condition. Elevated [CO_2] stimulates CO_2 assimilation (A) and intrinsic water use efficiency (iWUE) even under WD. Drought condition slightly changed stomatal conductance, transpiration rate and maximum quantum efficiency of photosystem II (PSII) regardless of [CO_2] compared to WW-plants. Total soluble amino acid concentration did not change significantly, while total phenolic compounds concentration decreased under e[CO_2] regardless of water regimes. The combination of e[CO_2]+WD increased the 5-O-caffeoylquinic acid (5-CQA) and caffeine amounts by 40-day when compared to a[CO_2]+WD plants. Altogether, these results suggest that e[CO_2] buffers mild-drought stress in young *C. arabica* by increasing A, iWUE and stimulating changes in the leaf contents of 5-CQA and caffeine.

Keywords: climate change, water deficit, photosynthesis, specialized metabolites, coffee

INTRODUCTION

The concentration of carbon dioxide ([CO_2]) in the atmosphere is one of the environmental factors of the greatest influence on plant development. CO_2 emissions have gradually increased in recent decades as a consequence of anthropogenic activities, with a current concentration around 415 ppm (NOAA, 2020). The increase in atmospheric [CO_2] contributes to the greenhouse effect and changes in the precipitation pattern, which could favor flood and more frequent drought

events depending on the location (IPCC, 2014). Water deficit represents the main abiotic stress to affect crop production worldwide. To cope with drought conditions, plant metabolism is adjusted including a range of morphological, physiological, biochemical and molecular changes (Seleiman et al., 2021). The initial effects of drought on plants are related to stomatal closure, decrease in leaf area, acceleration of leaf senescence and abscission aiming to control water status. However, these responses reduce the availability of CO_2 in the chloroplasts inhibiting photosynthesis and modifying carbohydrate supply for metabolism (McDowell et al., 2008). As drought severity increase biochemical restrictions and oxidative stress impair plant development, which can later cause plant death (Seleiman et al., 2021).

The isolated effect of elevated [CO_2] (e[CO_2]) on plant growth and development has been studied in a variety of species, especially in annual crops and soybean (Ainsworth and Rogers, 2007; Gamage et al., 2018). These works report a species-specific response, but in general e[CO_2] stimulates photosynthesis by increasing [CO_2] in the vicinity of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), and reduces stomatal conductance and water loss. Additionally, plant growth enhancement is often observed, since photorespiration and reactive oxygen species (ROS) synthesis are inhibited in those conditions (DaMatta et al., 2016). Therefore, it is hypothesized that e[CO_2] could mitigate the negative effects of drought stress on plants.

Although few studies have involved woody plants, some of them show a promising beneficial effect of e[CO_2] when exposed to abiotic stresses (Ramalho et al., 2013; Xu et al., 2015; Sobuj et al., 2018). In *Coffea arabica* L., an important commodity worldwide, it was recently demonstrated that e[CO_2] promoted higher carbon assimilation rate, water use efficiency and biomass under drought conditions when compared to plants exposed to drought and ambient [CO_2] in a greenhouse (Sanches et al., 2017). These responses were also related to lower photorespiration rates and higher hydraulic conductance by the increase of aquaporins transcripts, which contributed to a better performance of these plants under water deficit conditions (Avila et al., 2020a,b). In *C. arabica* and *C. canephora*, e[CO_2] improved photosynthetic efficiency by reducing energy dissipation and PSII activity inhibition of plants exposed to moderate and severe water deficit (Semedo et al., 2021). The mitigating role of e[CO_2] was similarly observed in *C. arabica* trees under field conditions exposed to seasonal water deficit (Sanches et al., 2020). The authors assigned this positive effect to the increased levels of soluble carbohydrates, organic acids and amino acids in leaves of plants subjected to e[CO_2].

Despite these important findings, the mechanisms underlying these responses, as well as the role of specialized metabolites, are poorly understood. The coffee tree has a complex phytochemical composition, including caffeine and chlorogenic acids (CGAs) which are both products of plant specialized metabolism that act in plant protection against biotic and abiotic stresses (Frischknecht et al., 1986; Mondolot et al., 2006; Leitão et al., 2008; Gebeyehu and Bikila, 2015). Among the purine alkaloids, caffeine is the most abundant, being found in all organs of coffee plants, with greater abundance in flowers and especially in young leaves, in which a great biosynthetic rate and concentration are noticed (Ashihara, 2006; Abrahão et al., 2010; Perrois et al., 2014; Sunarharum et al., 2014; Da Silva et al., 2018). Concerning phenolic compounds, the 5-O-caffeoylquinic acid (5-CQA) is the most abundant among the chlorogenic acids (CGAs) present in coffee plants, which is considered the main component of the phenolic fraction. 5-CQA is obtained by the esterification between quinic acid (esterification formed on the hydroxyl group at carbon 5) and caffeic acid (Campa et al., 2008). These substances are mainly involved with plant resistance and defense due to their antioxidant properties, acting particularly in ROS scavenging and, consequently, reducing the effects of oxidative stress. Chlorogenic acids seem to be involved in different abiotic stress responses, as observed by Ramakrishna and Ravishankar (2011). Furthermore, the amount of caffeine and chlorogenic acids are economically relevant due to their contribution to the coffee drink quality (Monteiro and Farah, 2012; Cheng et al., 2016).

Recently, the effect of increasing [CO_2] on caffeine concentration was investigated in leaves of *C. arabica* and *C. canephora* grown under field conditions (Vega et al., 2020). In this work, caffeine amount was negatively correlated with e[CO_2] in *C. canephora* (cv. Robusta), but no interaction was found between [CO_2] and caffeine in *C. arabica*. In coffee beans, it was reported higher concentration of chlorogenic acids and kahweol when plants were exposed to water deficit and e[CO_2] (Marcheafave et al., 2020). These two studies clearly showed that somehow e[CO_2] and drought stress will affect synthesis/degradation of specialized metabolites in coffee plants. Nevertheless, the mechanisms underlying this process remain unclear, as is whether or not these compounds will be relevant to the acclimation of drought-stressed coffee plants. Therefore, the present study aimed to investigate the combined effect of e[CO_2] and progressive water deficit on water relations, gas exchange and specific specialized metabolites in leaves of *Coffea arabica* L. in an early growth stage. Results related to photosynthesis, water status and specialized metabolites are discussed in an integrative way.

MATERIALS AND METHODS

Plant Material and Experimental Conditions

Seedlings of *Coffea arabica* cv. Catuaí Vermelho IAC-144 (3–4-month-old) obtained in a coffee nursery and having a similar growth pattern were transplanted into 7-liter pots containing Plantmax Café® (DDL Agroindustria Ltda) as substrate, with one plant per pot. After 20-day of acclimation in a greenhouse, plants were transferred to open-top chambers (OTC) installed inside a greenhouse at the Department of Physiology and Biochemistry of the Institute of Botany São Paulo, Brazil, for CO_2 treatment acclimation (ambient atmospheric [CO_2] – a[CO_2] = 400 ppm or elevated atmospheric [CO_2] – e[CO_2] = 800 ppm), as described by Sanches et al. (2017). The gas injection was applied through a compressed CO_2 cylinder attached to the

system. During this [CO_2] acclimation period, plants were daily watered with tap water and supplied with Hoagland and Arnon's nutrient solution (Hoagland and Arnon, 1950) once a week. After 30 days of [CO_2] acclimation period (plants about 7–8 expanded leaf pairs), half of the plants grown in a[CO_2] and e[CO_2] were subjected to water deficit (WD) by total water withholding, while the other half was maintained on a daily watering regime (WW) for 40 consecutive days, featuring the following treatments: a[CO_2]+WW, a[CO_2]+WD, e[CO_2]+WW and e[CO_2]+WD. The experiment was performed from October to December 2018 and only [CO_2] inside the OTCs was controlled. The air temperature and relative humidity (HMP45C-L, Campbell Sci.) and photosynthetic active radiation (Li-190R, LiCor) were monitored inside the OTCs over the experiment period. The environmental conditions inside the OTCs during the experimental period were as follows: photosynthetic photon flux density (PPFD) of 302 ± 36 μmol photons m^{-2} s^{-1}, temperature (day/night) of 27.7 ± 0.6°C/20.9 ± 0.5°C, relative humidity (day/night) of 63.9 ± 1.2%/82.9 ± 0.7%, a[CO_2] of 380 ± 41 ppm, e[CO_2] of 780 ± 28 ppm, and 12-h of photoperiod (**Supplementary Figures 1, 2**). During the experimental period, measurements of gas exchange, chlorophyll *a* fluorescence, water potential (Ψ_w), soil moisture and sampling of leaf material for further analysis were performed on day 0, 20, and 40 after water deficit imposition. All instantaneous physiological analysis took place in the morning from 06:00 am to 11:30 a.m.

Ecophysiological Analysis

Soil Moisture and Leaf Water Potential

The soil moisture was measured by the Time Domain Reflectometry (TDR) method, using a ML2-x Delta-T Devices model sensor (Theta-Probe, Cambridge, UK). Water potential (Ψ_w) was measured from 06-07 am using a Scholander pressure pump (model 1000, PMS Instrument Co) in fully expanded leaves of the third to the fourth pair from the apex immediately opposite to the leaves that were used for instantaneous photosynthetic measurements.

Gas Exchanges and Chlorophyll *a* Fluorescence

The instantaneous net CO_2 assimilation rate (A), stomatal conductance (g_s), leaf transpiration rate (E), and intercellular CO_2 partial pressure (Ci) were determined on fully expanded leaves of the third to fourth pair (from the apex and at the opposite side that Ψ_w was measured) using a portable infrared gas analyzer (IRGA model LC-SD Pro, ADC Bioscentific), under [CO_2] of 400 ppm and 800 ppm, according to plant [CO_2] condition, and at photosynthetic saturation light (PPFD of 600 μmol photons m^{-2} s^{-1}) (Sanches et al., 2017). The intrinsic water use efficiency (iWUE) was calculated as A/g_s. The chlorophyll *a* fluorescence emission was measured on dark-adapted (30 min) leaves according to the saturation pulse (Schreiber, 2004) using a portable fluorometer (OS5p Opti-Sciences, Hudson, NH, USA). The intensity and duration of the saturation pulse were 3,000 μmol m^{-2} s^{-1} and 1 s, respectively. The following parameters were assessed: minimum fluorescence (F_0), maximum fluorescence (F_m) and maximum quantum efficiency of photosystem II (PSII) [$F_v/F_m = (F_m\text{-}F_0)/F_m$)].

Biochemical Analysis

Mature leaves (third to fourth pairs from the apex) were taken in each sampling time (day 0, 20, and 40 after water deficit imposition) after the photosynthetic measurements and rapidly snap-frozen in liquid nitrogen followed by storage at −80°C until analysis. Later, the extract for biochemical analysis was performed as follows: leaf samples were freeze-dried, ground in a mill, incubated in 80% ethanol (v/v) for 48 h and constantly mixed. Afterwards, the solution was filtered to obtain the ethanolic extract and it was used in the subsequent analysis.

Total Phenolic Compounds and Total Soluble Amino Acids

The total soluble amino acids (TSA) was determined according to Yemm et al. (1955), using L-leucine (Fluka®) as a standard. The absorbance reading was performed on a spectrophotometer (Bel SPECTRO S05) at 570 nm. The concentration of total phenolic compounds (TPC) was quantified according to the Folin-Ciocalteau method (Singleton and Rossi, 1965), modified by Marinova et al. (2005), using gallic acid (Sigma) as a standard. Subsequently, TPC levels were determined in an ELISA microplate reader (KC4, Biotek Instruments) at 750 nm.

Caffeine and 5-O-Caffeoylquinic Acid

The quantification of caffeine and 5-O-caffeoylquinic acid (5-CQA) in the ethanol extracts obtained as described above was performed with samples after drying in a speed-vac system, solubilized in 1 mL of MeOH (Merck), filtered (Millipore filter 0.45 μm), stored in vials for analysis on an Agilent 1260 Liquid Chromatograph equipped with a 60 mm flow cell and photodiode array scanning spectrum detector (HPLC-UV/DAD), according to Tamayose et al. (2019). All analyzes were performed on a C18 reverse-phase column Zorbax Eclipse Plus (150 × 4.6 mm and 3.5 μm particle diameter) as a stationary phase. The mobile phase was composed of a mixture of eluents using a gradient system, started with a mixture of 90% H_2O acidified with 0.1% acetic acid and 10% acetonitrile (ACN) up to 6 min, 6–7 min, 10–15% ACN, and 7–20 min 15% ACN with a flow rate of 1.0 mL min^{-1} and injection volume of 1 μL of each sample. Chromatographic profiles were recorded at 254 nm, 280 nm, 325 nm and 352 nm. Standard curves were obtained with 3 μL of stock solutions of authentic standards of caffeine and 5-CQA in methanol, at concentrations of 1, 5, 10, 20, 40, 60, 100, 120, 150, 200, 300, and 400 μg mL^{-1}). The chromatographic profiles of the crude extracts, the ultraviolet spectra and commercial standards (Sigma) allowed identifying two metabolites present in the crude extracts as 5-CQA (t_R ∼3.84 min) and caffeine (t_R ∼5.23 min). Quantification of compounds was carried out by external standard method.

Statistical Analysis

The experiment was arranged in a completely randomized design, 2×2×3 factorial (two atmospheric [CO_2] concentrations—a[CO_2] and e[CO_2], two water regimes—well-watered and water-deficit and three sampling times—day

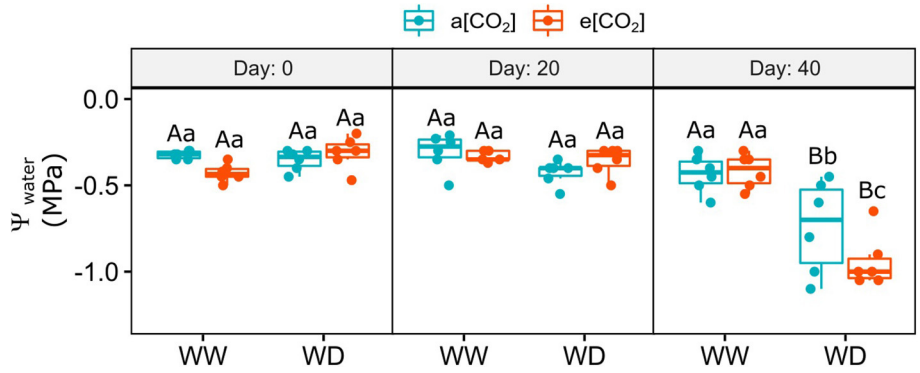

FIGURE 1 | Leaf water potential of *C. arabica* plants exposed to ambient [CO_2] under well-watered and water deficit (a[CO_2]+WW and a[CO_2]+WD, respectively) and elevated [CO_2] under well-watered and water deficit (e[CO_2]+WW and e[CO_2]+WD, respectively) conditions on day 0, 20, and 40 after water deficit imposition in the OTC. Boxes represent the median and first and third quartiles, symbols represent individual samples (n = 6–5 biological replicates). Different capital letters represent significant differences between sampling day in the same water and [CO_2] condition, whereas different lowercase letters represent significant differences between water and [CO_2] conditions in the same sampling day according to Tukey's test at 5% of probability ($P \leq 0.05$).

0, 20, and 40 after water withholding onset) with 5–6 replicates per treatment, each one represented by one plant per pot. The data obtained were submitted to analysis of variance (ANOVA) and the contrast between averages was further evaluated by Tukey's test at 5% of probability ($P \leq 0.05$). Figures were created in R (version 4.0.2) and RStudio (version 1.3.959). Box and whiskers plots were prepared using ggplot2 package; boxes show medians and first and third quartiles (25th and 75th percentiles), and whiskers extend from the hinge to the largest or smallest value, no further than 1.5 times. The statistical analyses showed in the box plots were performed using SigmaPlot 12.0 (Systat Software, San Jose, USA). The multivariate approach by principal component analysis (PCA) was performed using the R FactoMineR and factoextra packages (Kassambara and Mundt, 2017; Husson et al., 2020) in order to verify the behavior of samples and variables. The decompose of additive time-series was performed in R (version 4.0.2) and RStudio (version 1.3.959) using the commands "ts()" and "decompose()" from the preinstalled stats package.

RESULTS

Water Deficit Drastically Decreased Soil Moisture and Slightly Reduced Leaf Water Status Regardless [CO_2]

Soil moisture was measured every 10 days after water withdrawal imposition (**Supplementary Figure 3**). In the first 10 days, the water content in the substrate of the water-deficit (WD) pots did not change compared to the well-watered (WW) ones. After that, soil moisture progressively reduced from day 20 to 40 (∼58 and 89%, respectively) regardless [CO_2], compared to WW pots. The impact of WD on leaf water potential was observed only on day 40 after WD imposition, which decreased from −0.4 at WW conditions to −0.7 in a[CO_2]+WD plants and −0.9 in e[CO_2]+WD plants (**Figure 1**).

Elevated [CO_2] Augmented CO_2 Assimilation and Water Use Efficiency With Little Impact on Stomatal Conductance and PSII Activity

On day 0 (i.e., 30 days after e[CO_2] acclimation and before water restriction), CO_2 assimilation and intrinsic water use efficiency increased 3- and 3.9-fold, respectively, and remained higher in WW condition relative to a[CO_2] plants (**Figures 2A,D**). Water deficit reduced these parameters in e[CO_2]+WD plants on day 20 and 40 after the stress onset, relative to e[CO_2]+WW, but they were still higher than a[CO_2]+WD, especially on day 20 (**Figures 2A,D**). Stomatal conductance and transpiration rate were affected by WD only on day 40 after stress imposition irrespective [CO_2], which reduced by around 60 and 51%, respectively, compared to WW plants (**Figures 2B,C**). The gas exchange measurements of a[CO_2] plants was not affected by WD relative to WW plants (**Figure 2**). The stomatal conductance of all plants on day 20 was higher than day 0 and 40, probably as a consequence of lower temperature and higher air relative humidity on this day (**Supplementary Figures 1, 2**). The intercellular CO_2 partial pressure (Ci) increased over the experiment in e[CO_2]-enriched plants when compared to a[CO_2] ones and it was not affected by WD (**Supplementary Figure 4**).

The chlorophyll *a* minimum and maximum fluorescence decreased similarly in plants acclimated to e[CO_2] on day 0 of water shortage, mainly under WD, irrespective to [CO_2], when compared to a[CO_2]+WW plants (**Figures 3A,B**). On day 20, the levels of these parameters in all treatments returned to the levels found in a[CO_2]+WW on day 0, but decreased equally in all treatments on day 40, possibly as an effect of the environmental condition variations (**Figures 3A,B**; **Supplementary Figures 1, 2**). Despite no statistical difference between treatments, on day 40 it was observed that F_0 was quite variable in e[CO_2]+WD plants. The maximum quantum efficiency of PSII did not change in the first two sampling times

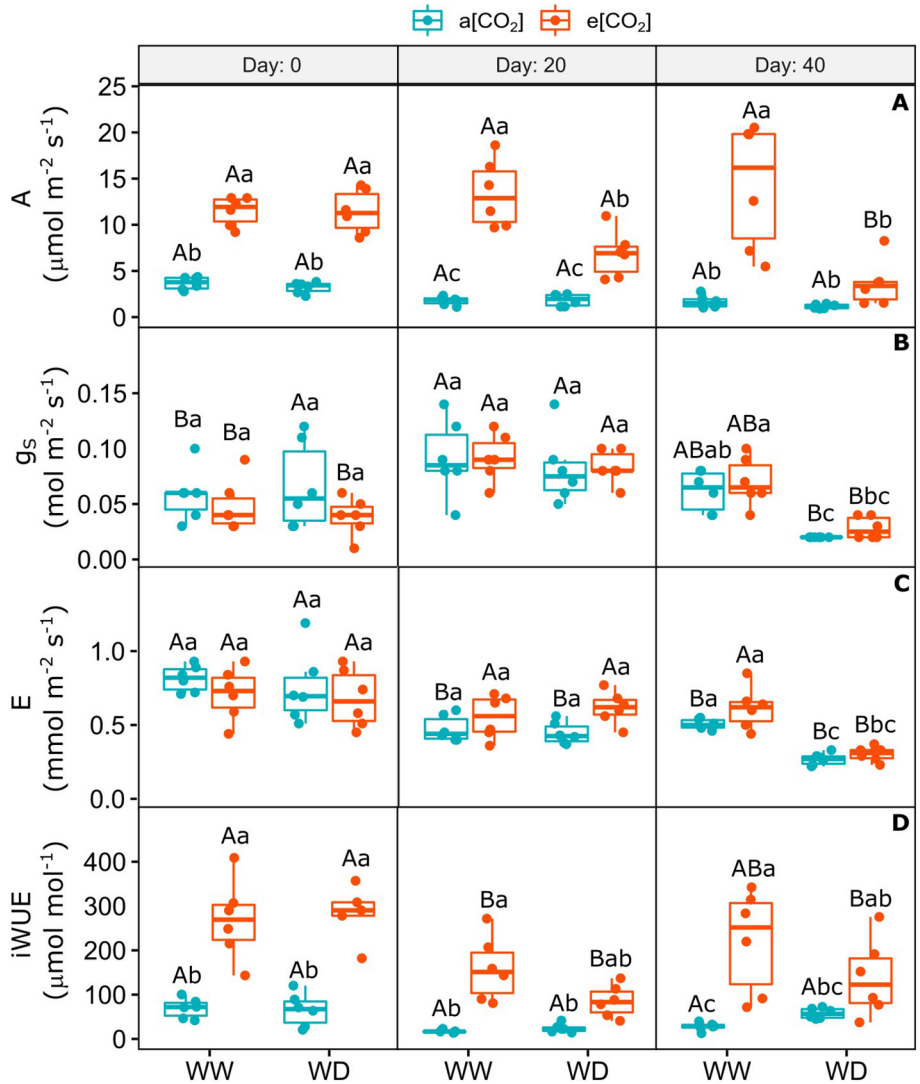

FIGURE 2 | Gas exchange of *C. arabica* plants exposed to ambient [CO_2] under well-watered and water deficit (a[CO_2]+WW and a[CO_2]+WD, respectively) and elevated [CO_2] under well-watered and water deficit (e[CO_2]+WW and e[CO_2]+WD, respectively) conditions on day 0, 20, and 40 after water deficit imposition in the OTC. **(A)** Net CO_2 assimilation, **(B)** Stomatal conductance, **(C)** Transpiration rate, and **(D)** Intrinsic water use efficiency. Boxes represent the median and first and third quartiles, symbols represent individual samples (n = 6–5 biological replicates). Different capital letters represent significant differences between sampling day in the same water and [CO_2] condition, whereas different lowercase letters represent significant differences between water and [CO_2] conditions in the same sampling day according to Tukey's test at 5% of probability ($P \leq 0.05$).

and slightly decreased (18%) in e[CO_2]+WD plants compared to and e[CO_2]+WW plants (**Figure 3C**).

Elevated [CO_2] Decreased Total Phenolic Compounds Amount and Increased the Content of 5-O-Caffeoylquinic Acid and Caffeine in Leaves of Drought-Stressed Plants

The concentration of total soluble amino acids was variable and did not change between treatments over the experimental period, despite the tendency to increase in e[CO_2]+WD plants on day 20 and 40 (**Figure 4A**). The amount of total phenolic compounds (TPC) increased over time in a[CO_2]+WW plants (**Figure 4B**). While e[CO_2] did not change the concentration of TPC on day 0, it was equally decreased by e[CO_2] and water deficit on day 20, when compared to a[CO_2]+WW plants (**Figure 4B**). On day 40, this response remained, especially in e[CO_2] plants, regarding a[CO_2]+WW plants (**Figure 4B**).

Elevated [CO_2] did not change the amount of 5-O-caffeoylquinic acid (5-CQA) and caffeine on day 0 and 20, despite the large variation between treatments (**Figure 5**). On day 40, the combination of e[CO_2] and WD increased the concentration of 5-CQA, when compared to e[CO_2]+WW and a[CO_2]+WD plants (**Figure 5A**). Moreover, e[CO_2] and WD treatment stimulated the accumulation of caffeine regarding

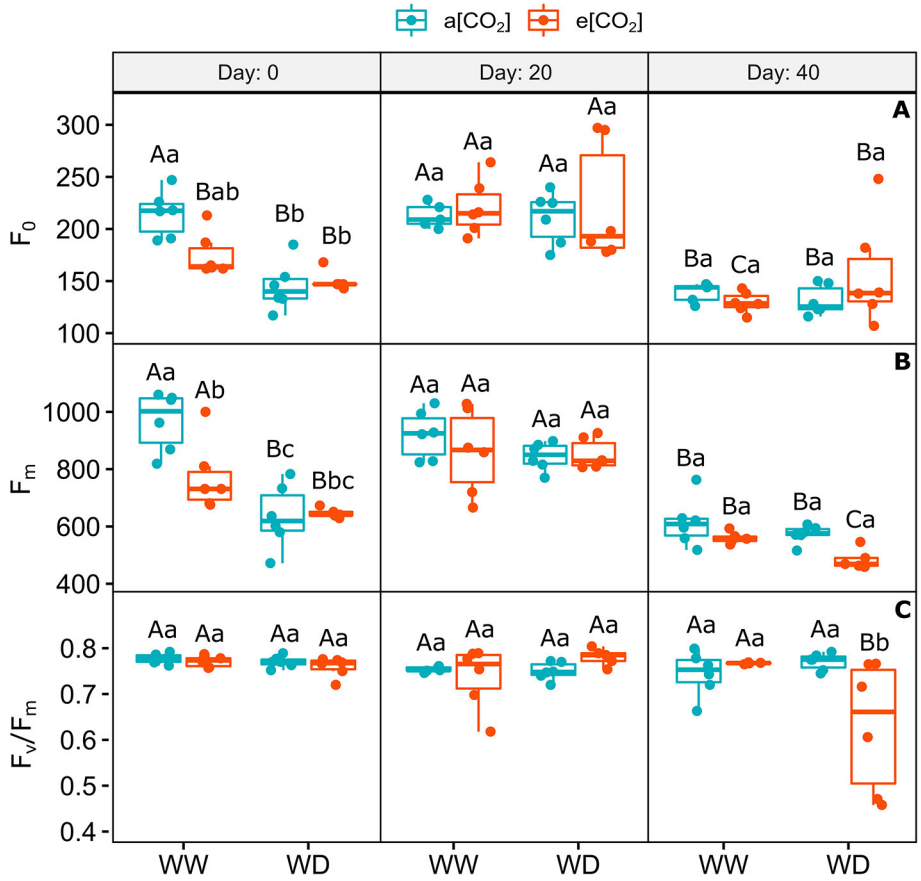

FIGURE 3 | Photochemical parameters of *C. arabica* plants exposed to ambient [CO_2] under well-watered and water deficit (a[CO_2]+WW and a[CO_2]+WD, respectively) and elevated [CO_2] under well-watered and water deficit (e[CO_2]+WW and e[CO_2]+WD, respectively) conditions on day 0, 20, and 40 after water deficit imposition in the OTC. **(A)** Chlorophyll *a* minimum fluorescence, **(B)** Maximum fluorescence, and **(C)** Maximum quantum efficiency of PSII. Boxes represent the median and first and third quartiles, symbols represent individual samples (n = 6–5 biological replicates). Different capital letters represent significant differences between sampling day in the same water and [CO_2] condition, whereas different lowercase letters represent significant differences between water and [CO_2] conditions in the same sampling day according to Tukey's test at 5% of probability ($P \leq 0.05$).

e[CO_2]+WW plants, but it was similar to a[CO_2] plants irrespective of water regime, on day 40 (**Figure 5B**).

Specialized Metabolites as Affected by e[CO_2] and Water Deficit

A multivariate approach was performed using PCA analysis aiming to better understand the data set variation. **Figure 6** shows the distribution of variables determined in this study, including all treatments and sampling times. This analysis indicates that leaf water potential, F_m, soil moisture, intrinsic water use efficiency, stomatal conductance and transpiration rate were the main relevant variables for principal component 1 and 2 (**Supplementary Figure 5**). Additionally, 5-CQA and caffeine showed a distinct behavior when compared with other physiological and biochemical variables, such as photosynthetic rate and chlorophyll *a* fluorescence, and total soluble amino acids (**Figure 6**).

The same analysis was carried out in each sampling point to verify the distribution of treatments and variables (**Figure 7**). On day 0 and 20, there was no clear distribution pattern between treatments, but samples from e[CO_2] tend to behave differently from a[CO_2] samples (**Figures 7A,B**). On day 40, plants under water deficit were separated from well-watered plants (**Figure 7C**). Despite no clear separation between a[CO_2]+WD and e[CO_2]+WD groups was found, there was a tendency of different distribution supported by a similar behave of 5-CQA and caffeine in e[CO_2]+WD plants, whereas amino acids content seems to be important to a[CO_2]+WD plants (**Figure 7C**).

DISCUSSION

Coffee plants cultivated under natural conditions are constantly challenged by abiotic stresses (DaMatta and Ramalho, 2006; DaMatta et al., 2018; Sanches et al., 2020). However, how the predictable environmental conditions related to high [CO_2] and

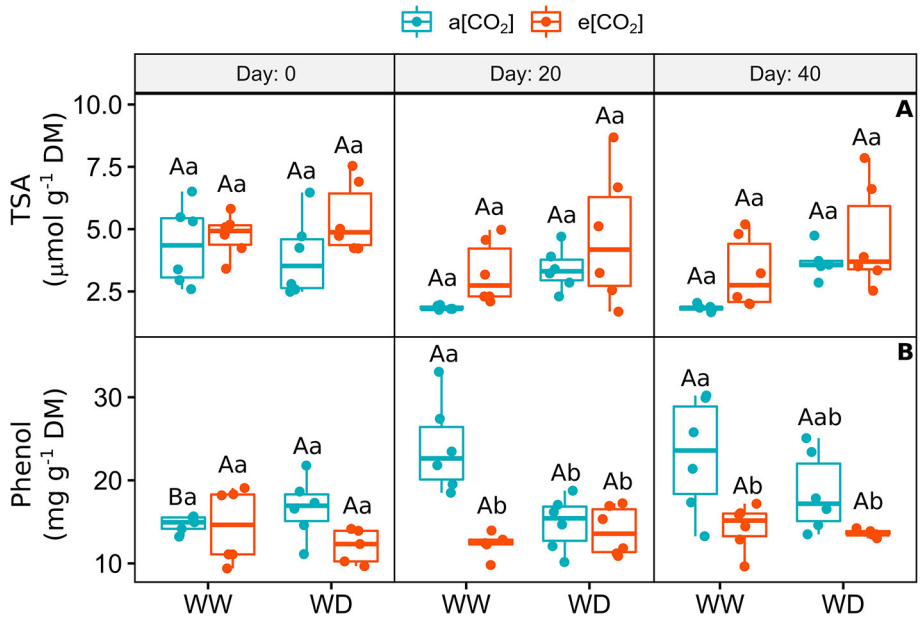

FIGURE 4 | (A) Content of total soluble amino acids and **(B)** Total phenolic compounds in leaves of *C. arabica* plants exposed to ambient [CO_2] under well-watered and water deficit (a[CO_2]+WW and a[CO_2]+WD, respectively) and elevated [CO_2] under well-watered and water deficit (e[CO_2]+WW and e[CO_2]+WD, respectively) conditions on day 0, 20, and 40 after water deficit imposition in the OTC. Boxes represent the median and first and third quartiles, symbols represent individual samples (n = 6–5 biological replicates). Different capital letters represent significant differences between sampling day in the same water and [CO_2] condition, whereas different lowercase letters represent significant differences between water and [CO_2] conditions in the same sampling day according to Tukey's test at 5% of probability ($P \leq 0.05$).

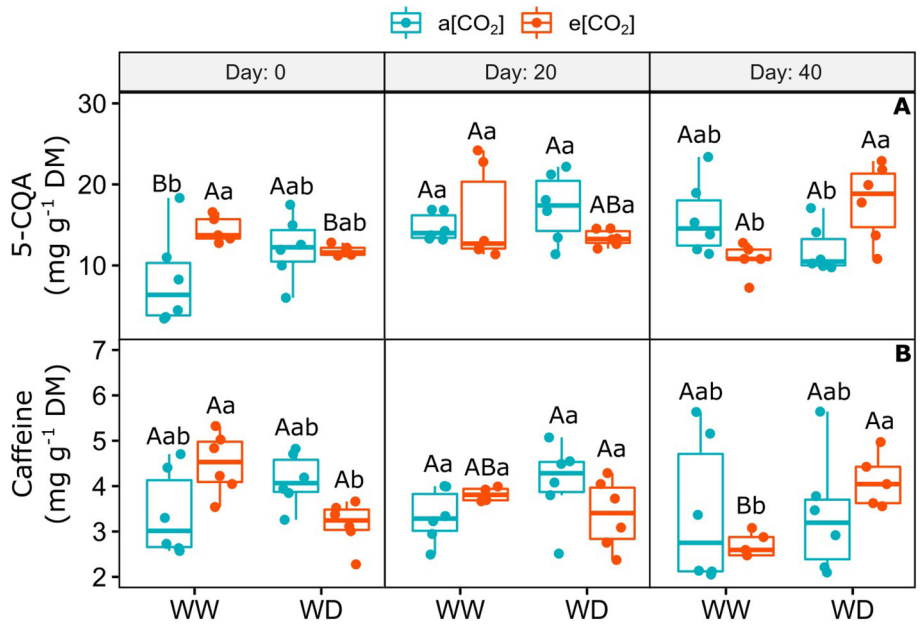

FIGURE 5 | (A) Content of 5-O-caffeoylquinic acid and **(B)** Caffeine in leaves of *C. arabica* plants exposed to ambient [CO_2] under well-watered and water deficit (a[CO_2]+WW and a[CO_2]+WD, respectively) and elevated [CO_2] under well-watered and water deficit (e[CO_2]+WW and e[CO_2]+WD, respectively) conditions on day 0, 20, and 40 after water deficit imposition in the OTC. Boxes represent the median and first and third quartiles, symbols represent individual samples (n = 6–5 biological replicates). Different capital letters represent significant differences between sampling day in the same water and [CO_2] condition, whereas different lowercase letters represent significant differences between water and [CO_2] conditions in the same sampling day according to Tukey's test at 5% of probability ($P \leq 0.05$).

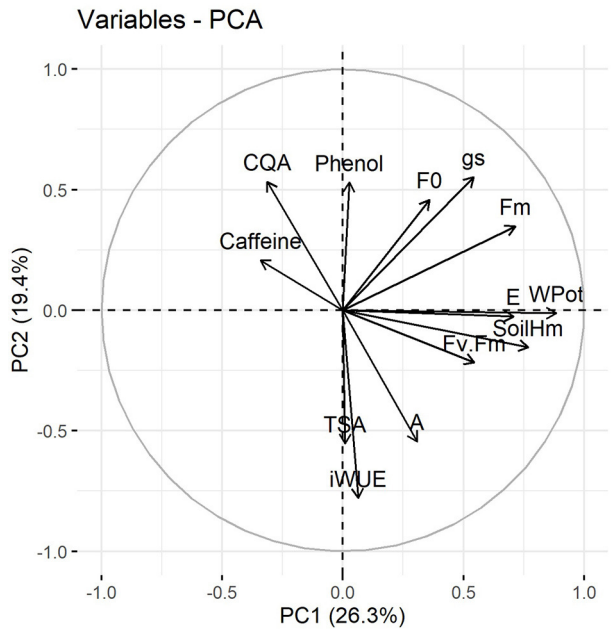

FIGURE 6 | Principal component analysis of physiological and biochemical variables of *C. arabica* plants exposed to ambient [CO_2] under well-watered and water deficit (a[CO_2]+WW and a[CO_2]+WD, respectively) and elevated [CO_2] under well-watered and water deficit (e[CO_2]+WW and e[CO_2]+WD, respectively) conditions on day 0, 20, and 40 after water deficit imposition in the OTC. Numbers in parentheses give the per cent variation explained by the first and second principal component. Variables abbreviation: CQA, 5-CQA; Phenol, Total phenolic compounds; WPot, Leaf water potential; Fv.Fm, F_v/F_m; SoilHm, Soil moisture.

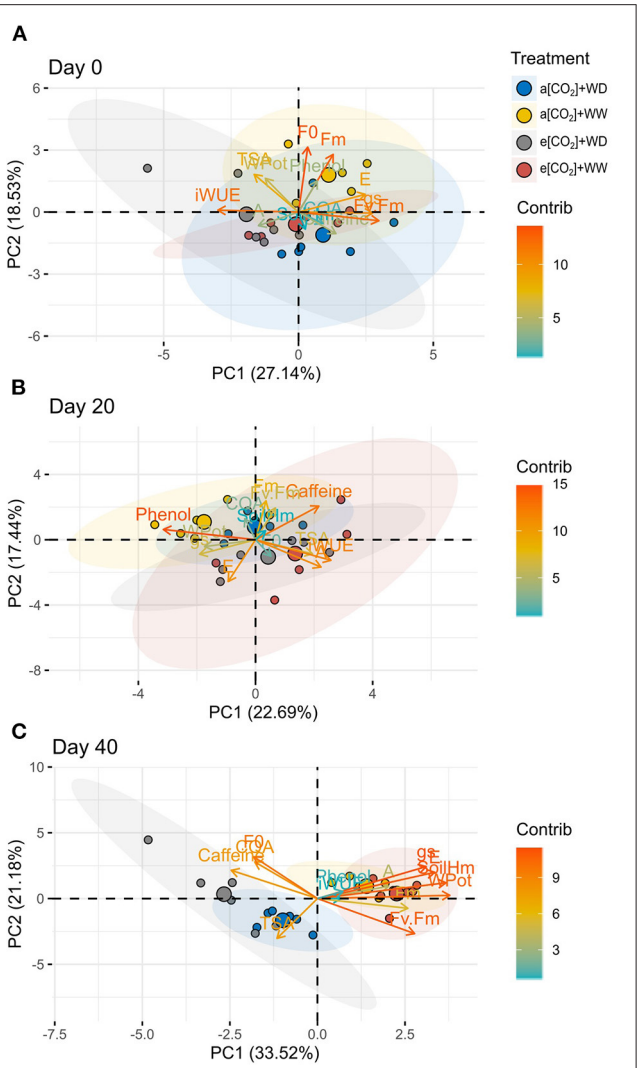

FIGURE 7 | Principal component analysis of physiological and biochemical variables and treatments of *C. arabica* plants exposed to ambient [CO_2] under well-watered and water deficit (a[CO_2]+WW and a[CO_2]+WD, respectively) and elevated [CO_2] under well-watered and water deficit (e[CO_2]+WW and e[CO_2]+WD, respectively) conditions on day **(A)** 0, **(B)** 20, and **(C)** 40 after water deficit imposition in the OTC. Numbers in parentheses give the per cent variation explained by the first and second principal component. The color and distribution of each variable depending on their contribution to each principal component. Variables abbreviation: CQA, 5-CQA; Phenol, Total phenolic compounds; WPot, Leaf water potential; Fv.Fm, F_v/F_m; SoilHm, Soil moisture.

the frequent drought events will affect coffee plants metabolism are not fully understood. In this study, we assessed whether elevated [CO_2] will ameliorate drought stress and how the combination of these environmental conditions will affect the metabolism of specialized metabolites in young *C. arabica* plants. The effects of water deficit in plants will depend on the stress level (duration, intensity, and frequency), other environmental conditions (temperature, relative humidity and vapor pressure deficit) and interaction with other stresses, soil characteristics, plant stage and ability to tolerate the stress (Seleiman et al., 2021). In this study, water deficit drastically decreased soil moisture, but leaf water potential decreased only to about −1 MPa after 40 days of water withdrawal (**Supplementary Figure 3**; **Figure 1**). This water potential level represents mild to moderate drought stress to coffee, which has a narrow stomatal conductance and an efficient water status control (Martins et al., 2019; Semedo et al., 2021). Despite the very dry soil condition, the environmental conditions related to air temperature and relative humidity and the light intensity inside the OTCs were optimum to coffee plants and did not aggravate the stress. Indeed, this stress condition slightly affected gas exchange with no severe impacts on the PSII apparatus (**Figures 2**, **3**).

Furthermore, elevated [CO_2] stimulated CO_2 assimilation, as a consequence of higher Ci (**Supplementary Figure 4**), and water use efficiency with no influence on stomatal conductance and transpiration rate, as reported by previous works (DaMatta et al., 2016; Sanches et al., 2017; Avila et al., 2020a). This response supports the hypothesis that the current atmospheric [CO_2] is limiting for coffee plants photosynthesis since they have very low stomata aperture and insensitivity to [CO_2] (DaMatta et al., 2016; Rodrigues et al., 2016). The gas exchange performance of plants exposed to e[CO_2] and WD were not significantly different from plants exposed to ambient [CO_2] and WD. However, higher medians were noted for these parameters on

plants subjected to e[CO_2] than a[CO_2] under drought. These results suggest that coffee plants, even during the early growth stages, have an efficient water status control to keep a basal photosynthetic rate and when combined with drought, e[CO_2] has the potential to buffer the negative effect of this stress on gas exchange.

Recently, it was demonstrated that coffee plants fertilized with e[CO_2] have higher photosynthesis than ambient [CO_2] under water deficit conditions in both greenhouse or field environments (Sanches et al., 2017, 2020). In this short-term experiment, considering the mild water deficit reached after 40 days in the WD plants (−1.0 MPa), the same pattern in gas exchange of coffee plants was observed. To better understand this behavior, we are carrying additional experiments subjecting the plants to a more intense water deficit associated with transcriptomic and phenotypic analysis. This approach aims to understand the implications of the intensity and duration of drought on the positive effect of e[CO_2] on plant photosynthesis, carbon metabolism and ultimately on coffee plant growth.

The influence of e[CO_2] in the presence or not of water deficit on PSII integrity was little (**Figure 3**). The slight decrease in F_v/F_m of e[CO_2]+WD plants on day 40 could be assigned to the variations of F_0, which was not statistically different from the other treatments. This response suggests that young coffee plants have an efficient capacity to dissipate excess energy in response to the imposed stress. Similar results were observed previously (Cavatte et al., 2012; Peloso et al., 2017; Semedo et al., 2021), where coffee plants submitted to different levels of water deficit did not show major changes in F_v/F_m, indicating that coffee plants exposed to that drought conditions may not have undergone photoinhibition. In fact, coffee plants seem to have an efficient protective thermal dissipation by activating the non-photochemical quenching, and a ROS scavenging system in the thylakoid membranes (Fortunato et al., 2010; Pompelli et al., 2010; Semedo et al., 2021). Hence, as it was observed in this study, a slight effect can be expected in the PSII activity of coffee plants exposed to mild water deficit and sub-saturating light conditions.

This study shows that e[CO_2] probably increase the availability of CO_2 next to Rubisco active sites, stimulating photosynthesis and buffer the negative effects of drought by increasing water use efficiency, in accordance with previous works (Avila et al., 2020b; Semedo et al., 2021). As mentioned above, *C. arabica* plants are drought resistant due to their low stomatal aperture and efficient water status control. This later can be improved by e[CO_2], since it was observed that e[CO_2]-fertilized plants kept hydraulic conductance and higher expression of aquaporin genes (Avila et al., 2020a). Moreover, we observed here that water deficit decreased the beneficial effect of e[CO_2] in the gas exchange plant performance, which can be possibly related to biochemical limitations once stomatal and photochemical restrictions did not happen.

Our results also showed no statistically significant difference in total soluble amino acids (TSA) between a[CO_2] and e[CO_2] plants, but these later presented higher medians mainly under water deficit conditions (**Figure 4**). Therefore, it is possible to infer that the synthesis of TSA may not be the main sink of photoassimilates in e[CO_2]-fertilized plants of *C. arabica*. Moreover, other solutes besides TSA may contribute to the osmotic adjustment of these plants under drought (Paixão et al., 2014; Avila et al., 2020a; Sanches et al., 2020).

In coffee, specialized metabolites are diverse and their amount varies according to plant development stage and tissue. These compounds are classified as specialized metabolites and act in many metabolic pathways. Besides, the role of these compounds is mainly related to antioxidant and plant defense responses, important under biotic and abiotic stresses (Baumann, 2006; Naikoo et al., 2019). The effect of elevated [CO_2] under well-watered and water-deficit conditions on the metabolism of the three major specialized metabolites in coffee was investigated here (**Figures 4**, **5**). The concentration of phenolic compounds increased over time in plants under normal conditions (a[CO_2]+WW), corroborating with previous studies that showed higher accumulation of these compounds in older leaves of *C. arabica* plants (Kristiningrum et al., 2016). In plants subjected to e[CO_2], despite water availability, the amount of TPC (total phenolic compounds) did not undergo major changes over time, but it was lower than a[CO_2] plants on day 40. It suggests that the carbon skeletons synthesized by the higher photosynthetic rates under e[CO_2] were probably mobilized to sustain other pathways, such as carbohydrate and amino acid synthesis and plant growth, as it was already demonstrated in coffee plants (Sanches et al., 2017; Avila et al., 2020b).

In fact, in an early development stage, rapid tissue growth is supported by a high synthesis/degradation of primary metabolites (Aharoni and Galili, 2011). However, the production and accumulation of specialized compounds are also noticed, especially in young leaves and beans from the upper layers, which are more exposed to intense light (Mondolot et al., 2006; Rakocevic et al., 2021). This response seems to be stimulated by e[CO_2] exposure and it is species-specific (Sallas et al., 2001, 2003; Matros et al., 2006; Rakocevic et al., 2021). In needles scots pine seedling, e[CO_2] did not affect TPC whereas in needles of Norway spruce seedlings it was reduced by e[CO_2] (Sallas et al., 2003). The authors related that this response may be involved with the higher growth rates in Norway spruce needles. Moreover, it was also demonstrated that e[CO_2] trend to increase specialized metabolites, but it can be restricted to particular compounds depend on plant species (Peñuelas and Estiarte, 1998; Sallas et al., 2003). Bearing in mind the growth stage and treatments imposed in this study, it is worth highlighting the tendency of increasing the concentration of 5-CQA throughout the experiment, especially in e[CO_2]+WD treatment. In that case, it can be proposed that the fertilizing effect of the additional [CO_2] observed in photosynthesis, even under WD, may favor vegetative growth and explain the gradual increase in 5-CQA levels under these conditions. In *C. canephora* plants, Mondolot et al. (2006) also observed an accumulation of 5-CQA, especially in young leaves.

A previous study showed that moderate drought alters caffeine content in *C. canephora* leaves (Kumar et al., 2015), which is decreased and the genes involved in its biosynthesis are

possibly downregulated. On the other hand, a tendency toward an increase in the caffeine content in the treatment e[CO_2]+WD, indicates a positive effect of e[CO_2] on the concentrations of this compound in leaves. Recently, an experiment with arabica coffee trees subjected to four CO_2 concentrations (300, 400, 500 and 600 ppm) under non-stressful conditions, showed that e[CO_2] did not alter significantly the caffeine accumulation in leaves (Perrois et al., 2014).

In our study, the multivariate analysis indicate that specialized metabolites show distinct behavior comparing the physiological responses (**Figure 6**). As pointed earlier it might be associated with the use the additional carbon for specialized molecules synthesis. Surprisingly, after 40 days of treatments, the effect of water deficit appears as the main factor to distinguish the samples (**Figure 7C**), explaining one-third of the total variation (PC1). From these observations, we can conclude that the period of experiment was enough to allow the accumulation of the specialized metabolite by water deficit imposition but too short to allow the compensation of elevated [CO_2], considering these variables. Further studies including a large-scale analysis of transcripts and metabolites, as well as more intense drought stress, will bring insightful contributions of future climatic scenarios in coffee production.

CONCLUSION

Collectively, our results demonstrated that e[CO_2] had a positive effect on photosynthetic rates and intrinsic water use efficiency, buffering the extension of negative effects of mild drought stress. Moreover, water deficit and e[CO_2] influenced the increased leaf concentration of 5-CQA and caffeine throughout the experimental period, which could play an important role in the vegetative phase of young coffee plants under this environmental condition. This work adds new insights about the short-term combination of e[CO_2] and progressive water deficit affect the leaf concentration of these two specialized metabolites during the early growth stage of *Coffea arabica* plants. However, more studies are necessary especially involving long-term exposure to e[CO_2] and drought and large scale analyses of transcripts and metabolomics to better understand the metabolism regulation and how coffee plants will cope with the predicted environmental scenario.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

ES and LT designed the study. ES and DD supervised and acquired funds for the research. IC and GM conducted the experiments. MF and DC performed formal analysis. AL performed data curation. IC and AL wrote the manuscript with the contribution of all authors. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.676207/full#supplementary-material

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Breeding for the Main Agricultural Farming of Arabica Coffee

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So far, the main Arabica coffee breeding programmes in Latin America have focused on the selection of varieties adapted to intensive full-sun farming systems. Meanwhile, little attention has been paid to breeding varieties specifically adapted to shade, which is the main characteristic of agroforestry systems (AFS). Yet the specialty coffee sector is currently expanding and seeking specific sensory qualities related to exotic varieties and no breeding programme exists to create new varieties for this type of market. Two trials were set up: one in full sun and the other under shade. F1 hybrids and their parents (pure lines and Ethiopian accessions) were studied in a factorial-crossing design to measure tree volume, yield (3 years), bean size, the bean NIRS signature and the final cupping score. Bean size and the final cupping score seemed to be relatively unaffected by shading. Strong maternal heritability was observed for bean size. In the trials, F1 hybrids produced "75–80%" more than the maternal lines and "40–50%" more than the male parents in the shade trial and in the full-sun trial, respectively. By choosing the F1 hybrid, it is possible to increase productivity under both shade and full sun while simultaneously achieving good sensory qualities. Selecting a F1 hybrid for "specialty coffee" seems exceptional. This raises a fundamental issue concerning the maternal heritability of seed biochemical composition. We conclude that selection under shade is essential for the selection of varieties adapted to AFS.

Keywords: ***Coffea arabica*, farming systems, agroforestry systems, specialty coffee, F1 hybrids, Dwarf American pure lines, wild Ethiopian accessions**

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INTRODUCTION

Coffee agroforestry systems (AFS) in which coffee is grown in association with other trees on the same plot of land are widespread in many tropical countries. When AFS are properly managed, they can buffer climatic fluctuations, and benefit from biological and economic synergies, leading to sustainable land management and higher and more stable incomes for local stakeholders (mainly smallholder farmers) (DaMatta, 2004). Under shade, coffee beans are denser and far more flavor intense, notably with fine acidity and a pleasant aroma (Muschler, 2001). Unfortunately, coffee AFS productivity can be up to 15–30% lower than in full-sun systems (Vaast et al., 2006). It is very difficult to maintain the ideal level of shade as regulating shade by pruning the branches of the shade trees is expensive, meaning the shade percentage is usually more than 40%. Another important reason put forward to explain the lower productivity of coffee in AFS is the fact that both dwarf and tall varieties developed for intensive full-sun systems are used even though they are not suitable for AFS cropping conditions (Bertrand et al., 2011).

In the present study, we are currently at the breeding stage, in which, like in most genetic breeding programmes, we are selecting pre-candidates we will then transplant to a farming environment to analyze G × E interactions, including interactions between coffee trees and shade trees. Interactions with shade trees are complex because, in addition to shade effects, evapotranspiration and root interactions must also be taken into consideration. In the first stage of breeding, it is difficult to compare small numbers accurately while also accounting for the effects of competition with shade trees. However, it is possible to mimic the effects of shading by using artificial shade. The first question we aim to answer is, is it possible to select genotypes that produce the equivalent quantity and quality under a high shade percentage (>50%), as in full sun?

Of the large volumes of coffees produced under AFS and full-sun systems, an ever-increasing quantity is being sold as "specialty coffee." The Association of Fine Coffees (SCA, https://sca.coffee/), defines specialty coffee based on product quality (green beans, roasted beans or the prepared beverage) and by the quality of life that coffee can deliver to everyone involved in its cultivation (Rhinehart, 2009). Specialty coffees can be distinguished from mainstream coffee based on a variety of factors including quality, sustainability, and closer relationships with growers (Bacon et al., 2008). Unfortunately, the productivity of farms that produce so-called specialty coffees is not included. Specialty coffee roasters have understood that the choice of genotype has a huge impact on sensory quality and that old varieties such as Geisha or Typica or Bourbon are becoming keys to the market for the highest quality specialty coffees. Unfortunately, the varieties that produce the best coffees are also relatively unproductive or very susceptible to coffee rust and are therefore not very profitable for farmers (McCook and Vandermeer, 2015). On the other hand, the exceptional quality of a very small number of varieties is recognized. The second question we aimed to answer is, can new varieties be bred for the specialty market that combine exceptional quality and good productivity in shade and/or in full-sun conditions ?

To answer these questions, we conducted two trials, one in shady and one in full-sun conditions. To our knowledge, this is the first time that selection in full sun and selection under shade has been compared in coffee species but also in other species. We evaluated whether the performances were equivalent in the two trials. We used the same two factorial designs to compare the performance of pure Colombian lines, wild accessions from Ethiopia and F1 hybrids. A strong heterosis effect (20–70% increase in yield) has been observed when F1 hybrids were obtained by crossing dwarf American pure line varieties with wild Ethiopian accessions in full sun (Bertrand et al., 2005). In the present study, we wanted to check if heterosis is of the same amplitude in the shade. We assessed whether the selection was comparable between the two factorial trials and produced the same selected genotypes with constraints linked to three specifications. The first specification reflects the requirements of an intensive cultivation system close to that defined in the green revolution (small tree volume, large bean size and high productivity per hectare). Based on the different orientations in plant breeding proposed by Lammerts van Bueren et al. (2018), this orientation would be considered as "trait-based breeding." The second specifications reflect the requirements of the Specialty Coffee association (https://sca.coffee/). This orientation would be considered "corporate-based breeding," resulting in globally adapted? adaptable? cultivars that produce the highest profit. The third specifications reflect the requirements of agroforestry (productivity per hectare, tree volume, bean size, and beverage quality). This system would be considered as "Ecosystem-based breeding" aimed at developing varieties adapted to agroforestry, illustrated by the European Breedcafs project (https://www.breedcafs.eu/). The Breedcafs approach is based on the need to increase productivity without harming the environment and ensuring the sustainability of the agroecosystems (Campbell et al., 2014; Struik and Kuyper, 2014). The experimental design we used and results we obtained allow us to draw key conclusions regarding the selection of candidate varieties suitable for use in different farming systems and targeting different markets. Based on these conclusions, we make some recommendations for the organization of the coffee sector regarding breeding.

MATERIALS AND METHODS

Planting Material and Crossing Design

Our study was conducted using a factorial crossing design involving crosses between wild and cultivated parents. Hybrids were obtained by crossing three dwarf cv. Castillo pure lines (Alvarado-Alvarado et al., 2005a), with 10 tall wild Ethiopian forms (hereafter referred to as "Ethiopian accessions") pre-selected for yield and cup quality in the coffee collection established in Colombia. Crosses were established by directed pollination. Each cv. Castillo line received pollen from wild Ethiopian accessions. **Table 1** lists the 28 F1 hybrids evaluated, including three maternal lines and 10 male parents.

Two trials (hereafter referred to as "shade" and "sun") were conducted with the same design, one in full sun and the other one in a plot under a net allowing only 55% of the photosynthetic photon flux density. It is technically complicated to work in

TABLE 1 | Genotypes studied: 28 F1 hybrids (dwarf), 3 maternal pure lines (dwarf), 10 Ethiopian accessions (tall).

"Wild" Ethiopian male	Castillo pure lines—Female		
	CU1842	CX2385	CX2848
E554	X	X	X
E286	X	X	
E057	X	X	X
E054	X	X	
E114	X	X	X
E047	X	X	X
E069	X	X	X
E291	X	X	X
E464	X	X	X
E501	X	X	X

AFS conditions in the first stage of the breeding programme, which involves choosing from a large number of crosses those that can be used in the following selection stage. In our study, we mimicked the effect of an agroforestry system by reducing light intensity, which is one of the main effects of AFS, using artificial shading. Another difficulty we had to solve is that it is not possible to compare dwarf and tall genotypes in the same plot. In both trials, dwarf genotypes, female parents and hybrids were compared in one sub-plot and Ethiopian tall parents were grown together in another sub-plot. The number of trees per genotype was 10 to 20 plants with total randomization within each sub-plot.

The plants were grown at the Naranjal experimental station in the municipality of Chinchiná—Caldas—Colombia (4° 58 ′17 45 "N latitude and 75° 39 ′09, 88″ W longitude), at 1,381 m asl. The minimum, average, and maximum temperatures were 17.4, 21.2, and 26.8°C, respectively. The average relative humidity was 84.2%, and the average annual precipitation was 3,568 mm.

The plantation densities were 1.3 m × 1.5 m (i.e., 5,128 trees.ha^{-1}) for the dwarf plants and 1.5 m x 1.5 m (i.e., 4,444 trees.ha^{-1}) for the tall plants (i.e., the Ethiopian accessions). The soils in which the plants were grown received 1,000 kg.ha^{-1}year of N-P-K-Ca-Mg (18-3-10-8-0.5) and 250 kg.ha^{-1}year of N. The plants were grown with zero pesticide applications.

Traits Observed

The coffee tree canopy volume was estimated at 24 months by comparing the shape of the tree to a cone. The radius (r) in cm, calculated by taking the average of the two largest plagiotropic branches, and the total height of the tree in cm, was used to estimate the conical volume V (cm3) = 1/3 × π × r² × h (Bryant and Kothmann, 1979).

Yield was measured in kg/tree of fresh berries. Yield was estimated over three growing seasons (2013–2016).

The incidence of rust per plant was assessed in years 2 and 3 by visual inspection using a scale of 0–4, where 0 = absence of lesions; 1= sporulating lesions reaching 1 to 5% of the total leaf area; 2 to 3 = gradual increase in the number of diseased branches with sporulating lesions, and 4 = more than 50% of the leaf area affected (very susceptible cultivars may have lost their leaves before the observation date).

Samples of healthy ripe cherries belonging to each genotype were handpicked during the highest production period, between July and September 2014, in four harvest sessions. The cherries were then processed individually using the wet method (de-pulping, fermentation and drying) to obtain at least 200 g of green coffee beans with "11–12%" final moisture content. The green coffee samples were sieved (mesh size 14 to 20) according to the Colombian technical standard NTC 5248 (I*nstituto Colombiano de Normas Técnicas y Certificación* [Instituto Colombiano de Normas Técnicas y Certificación (ICONTEC), 2004]. Beans smaller than sieve mesh size 15 were discarded along with defective beans. For each tree, we evaluated the size of the green beans from 16 to 20, as exportable coffee must be at least 16/64 inches).

The sensory evaluation was performed according to the Specialty Coffee Association (SCA) cupping protocol by the Coffee Growers Committee tasting panel in Valle del Cauca (Colombia), composed of five Q-Grader tasters. To avoid fatigue, a maximum of 7 samples were compared at a time. A standard session consisted of six sets, with seven samples per set and three cups of each sample. The standard session was performed twice in randomized order for blind analysis. The final cupping scores of each sample were obtained by summing the individual scores in the fragrance/aroma, flavor, aftertaste, acidity, body, balance, sweetness, uniformity, cleanliness, overall, and defects categories.

NIRS Phenotyping

The NIRS technique can be used to analyse coffee products as it can extract considerable information on the biochemical composition of the product. In a previous study (Posada et al., 2009), we showed that NIRS-based inter-variety relationships were stable across environments. In the present study, we tested whether the Euclidean distances between the NIRS signatures of the genotypes were the same under shade and in full sun.

NIR reflectance spectra were collected using a scanning monochromator NIR systems spectrophotometer (model 6500, Perstorp Analytical Inc., 1201 Tech Road Silver Spring, MD, USA) controlled by NIRS2 (4.0) software (Intrasoft Intl., LLC, RD109, Sellers Lane, Port Matilda, PA, USA). The analyses were performed on 50 gm aliquots of green coffee after grinding to <0.5 mm. Each sample represented one tree and each tree was analyzed. For each sample, an NIR spectrum was acquired in reflectance (R) mode, where R represents the reflectance energy in the 4,000–9,090 cm^{-1} range in 7 cm^{-1} steps. The log ($1/R$) absorbance spectrum was obtained by the mean of those measurements and comparison with the reference. The mean quadratic error, estimated from two subsamples (two distinct samplings of the same sample) based on the raw spectrum (log $1/R$), was under 300 μabs, which is below the manufacturer's specifications and indicated satisfactory repeatability of the spectral measurement. Given these results, a single spectrum was acquired per sample.

Data Analysis

JMP, XLSTAT, and R software packages were used for the statistical analyses.

Comparison of Hybrids and Parental Populations

To evaluate the performances of the three populations (Ethiopian accessions, Castillo pure lines and F1 hybrid population), data for each population grown under shade and in full sun were analyzed using one-way analysis of variance (ANOVA), followed by a Tukey's test at $P \leq 0.05$.

Comparison of Genotype Intra Trial and Inter Trial Variability

The performances of the genotypes in each trial were evaluated by ANOVA. To compare inter-trial performance, we calculated the correlation between the two trials using the LSmeans calculated for each genotype, and to compare the performance of the same genotype in the two trials, we used the non-parametric Wilcoxon rank-sum test.

Heritability Estimations

Analyses of variance of the full factorial mating design using a two-way cross-classification model were carried out according to the Henderson III procedure for shade and sun conditions. The R package lme4 was used (Bates et al., 2015). We used the following random effects model:

$$Y_{ijk} = \mu + M_i + F_j + (MF)_{ij} + E_{ijk}$$

where μ is the general mean, M_i is the random effect of the i^{th} female, F_j is the random effect of the j^{th} male, $(MF)_{ij}$ is the female x male interaction, and $E_{ijk}$is the within-family deviation of the k^{th} individual within the$(ij)^{th}$ female x male combination. We estimated the variance components (including maternal and paternal variance) and narrow-sense heritability for the main traits (yield, final cupping score, bean size).

The estimated phenotypic variance is free from any genetic model and is given by:

$$\hat{V}P = \hat{\sigma}^2_M + \hat{\sigma}^2_F + \hat{\sigma}^2_{(MF)} + \hat{\sigma}^2_E$$

As usual, the estimates of narrow-sense and broad-sense heritability are given by:

$$\hat{h}^2_n = \frac{\hat{V}A}{\hat{V}P}$$

and

$$\hat{h}^2_b = \frac{\hat{V}A + \hat{V}D}{\hat{V}P}, \text{ respectively.}$$

Stability of the Genotype Performances Across Plots

Chemometric processing initially consisted of a principal component analysis (PCA) based on the spectra on the 4,000–9,090 cm^{-1} segment. Factorial scores of principal components (PCs) showing an eigenvalue higher than 1 were used to create clusters using the Euclidean distance and the *k*-means algorithm (Posada et al., 2009). We tested the number of clusters to be created, i.e., from 2 for sun to 6 for shade conditions, to test the robustness of the variety types? We compared two approaches to test the stability of the performances of the coffee variety across plots, the final cupping score and the NIRS signature. The pseudo *r* of correlation was calculated by comparing the two Euclidean distance matrices using a Mantel test, along with comparison of the associated probabilities. The procedure that gave the highest pseudo *r* associated with the greatest probability was considered to be the most efficient.

Selection Based on Farming System Specifications

In the first approach, selection was based on specifications for intensive full-sun farming systems (which correspond to the conventional specifications used by the main coffee breeding programme in Latin America). Selection was based on three traits: tree volume, yield and bean size. In the second approach, selection was based on the final cupping score and productivity, which is the main trait of interest for the specialty coffee market. In the third approach, selection was based on AFS specifications with the following threshold: yield under shade at least equivalent to the best pure line grown in full sun; a final cupping score higher than 83/100; a tree volume lower than 40% of the largest pure line grown in full sun; a green bean size at least equivalent to the performance of the maternal line producing the smallest beans.

Clustering Genotypes in the Two Plots for Further Selection in Different Farming Systems

Rather than select the genotypes, we clustered the genotypes based on the Euclidean distance between varieties based on the yield, bean size, tree volume and the NIRS signature values. The information of the NIRS spectra was reduced by using the scores of genotypes on the main first factor resulting from a discriminant analysis of the six main PCs.

RESULTS

Performance of Parent Populations and Hybrids

The performances of the three populations were monitored in the shade and full-sun trials. The average production under shade was more than 40% lower than in full sun.

In both tests, the hybrids gave the best performance (**Figure 1**). They produced 75–80% more than the maternal lines and 40–50% more than the male parents in the shade and sun trials, respectively.

The seed produced by females and F1 hybrids were significantly larger than those produced by Ethiopian males in both shade and sun trials. However, no significant differences between females and F1 hybrids were observed in the two trials. The tree volume of the male parents appeared to was much larger than that of the hybrids or female lines. There was no significant difference in tree volume between the female lines and F1 hybrids in the shade and sun trials; nor was there any difference in tree volume between female lines (dwarfs) and F1 hybrids (also dwarfs) in the two conditions.

Surprisingly, female cultivars that had been specially selected for rust resistance showed significantly more symptoms than the Ethiopian genotypes or F1 hybrids. The final cupping score of males was significantly higher ($P < 0.01$) than the scores of F1 hybrids and female lines in both the shade and sun trials. No significant differences were found between hybrids and females in the two trials.

Comparison of Genotypic Performance Between the Two Trials

The linear regression for bean size genotypes under shade relative to the bean size in full sun was significant ($P < 0.001$) and highly predictive, with an r^2 of 0.68. For yield, the regression was significant ($P < 0.01$), with an r^2 of 0.42, even though this cannot be considered as predictive. We compared the performance of each genotype between shade and full sun (**Data sheet 1**) using the non-parametric Wilcoxon rank-sum test. Most genotypes

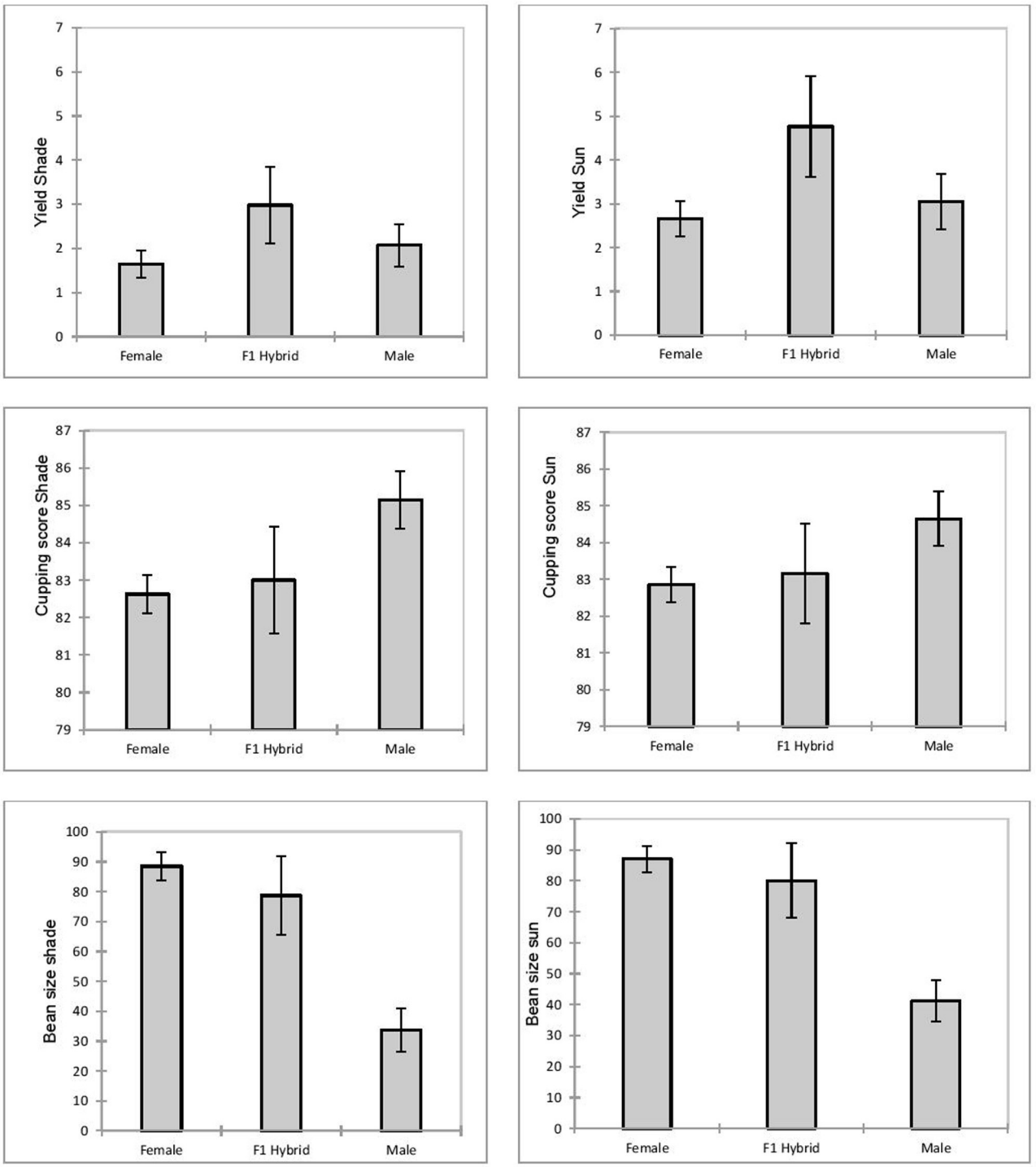

FIGURE 1 | Comparison of yield (expressed as kg of green coffee beans per plant), final cupping score (out of 100), bean size (percentage of bean size 16–20), in female, male and F1 hybrids in shade and full-sun plots.

produced significantly higher yields in full sun than under shade. We identified five genotypes whose performance did not differ significantly in the two conditions (E464, E057, E054, E047, and CX2385 × E291). The last two produced the same quantity of cherries in the shade as in the sun. Concerning the size of the beans, the performances of the great majority of the genotypes were identical in full sun and under shade. Seven genotypes (E501, E291, E286, E114, E054, E057, and CX2385 × E057)

showed a significant difference in favor of sun. Finally, the Ethiopian E047 and E554 produced significantly larger seeds under shade than in the full sun. The tree volume of all the genotypes was identical under shade and in full sun, except for the hybrid CX2848 × E114 whose tree volume was larger under full sun and the majority of the Ethiopian genotypes (E 464, E069, E291, E057, E114, E286, E047, E054) whose tree volumes were bigger when grown under shade (data not shown). Regarding the final cupping score, four genotypes scored better in full sun (CU1842, CU1842 x E464, CU1842 × E501, and the Ethiopian E501), while five genotypes grown under shade (E464, E069, E047, and the hybrids CX1842 × E069 and CX2385 × E069) often received higher scores.

Heritability of Main Traits

The regression of the mid-parent on hybrids for bean size was significant under shade ($r^2 = 0.25$) and null in full sun ($r^2 = 0.06$). However, we observed a strong maternal effect of this trait with a maternal heritability of between $h^2 = 0.69$ and 0.37, respectively, for shade and sun.

For the final cupping score, we observed a significant r^2 under shade and a non-significant r^2 in full sun (0.25 and 0.15, respectively) between the mid-parent performances and the hybrid performances. Female and male heritabilities for the final cupping score were close to zero, ranging between $h^2 = 0.04$ and 0.10 in full sun and under shade. Finally, the regression for the mid-parent regarding observed hybrid yield was null under shade and in full sun. The maternal or paternal heritabilities for yield were close to zero (h^2 female = 0.00 and 0.11, respectively, under shade and in full sun and h^2 male = 0.07 under shade and 0.23 in full sun). For yield, the mid-parent value did not appear to be a useful guide for the selection of parents to test combinations of hybrids.

Comparison of the Stability of the NIRS Genotypic Signature Under the Two Conditions

The PCA based on all the NIRS spectra in the sun trials produced a similar pattern. The first axis (PC1) separated the majority of the Ethiopian parents from the maternal parents (Castillo pure lines) (**Supplementary Figure 2**). The F1 hybrids were located midway between the two parental populations in full sun or under "shade."

We then performed a K-means analysis based on the first 15 PCs. We established three robust clusters corresponding to the three sub-populations (namely Ethiopian accessions, Castillo lines and F1 hybrids) confirming the PCA findings. We then used discriminant analysis to explain and predict the membership of observations according to their sub-groups. The first 15 factors that explained more than 99% of the total variance and had eigenvalues of over one were used to calculate the discriminant function models. We obtained a well-classified rate of around 90%, i.e., 91% per type in full sun and 90.3% under shade. We found same clustering in three subgroups for the NIRS signature. This stability shows that the two environmental conditions (sun and shade) did not significantly affect the interrelations between the NIRS signature of the genotypes.

Comparison of the Stability of the Euclidean Distance Matrices Under the Two Conditions

We compared the two distance matrices, sun and shade, using a Mantel test on the Euclidean matrix distances between genotypes using the factorial scores of the four PCs calculated in (A) on the phenotype values for yield, bean size, tree volume and final cupping score; in (B) on the phenotype values for yield, bean size, tree volume and NIRS signature. For the NIRS spectra, we used the genotype scores on the main first factor derived from a discriminant analysis of the 15 main PCs. We showed that the pseudo *r* of correlations obtained by comparing the two distance matrices based on a Mantel test and *p*-value was highly significant in both cases. Hence, the relative distances between genotypes were highly stable in both conditions when we used phenotyping on the four target traits (yield, bean size, tree volume and final cupping score) or an "extract" of the NIRS signature instead of sensory analysis. Use of the NIRS signature produced a higher result than that obtained using sensory analysis since the r obtained was higher (pseudo $r = 0.801$ using the NIRS signature vs. $r = 0.716$ using the final cupping scores).

Selection for Full Sun

The selection of the best candidates was based on a set of specifications whose priorities differ depending on the farming system. From previous results, it appeared? Previous result showed? that the female varieties, which are among the best lines currently disseminated in Colombia, were distinguished from the other genotypes by the remarkable size of their seeds. Indeed, this was a key criterion put forward by breeders in Colombia, the other criterion being tree volume, to adapt the varieties to an intensive full-sun system, which is the predominant farming system in Colombia. The three female varieties had a small volume, i.e., a trait that is very highly selected by breeders in Latin America. Highlighting this trait along with bean size revealed two distinct groups: females and F1 hybrids on the one hand, and male genotypes on the other in the full sun plot and the shade plot (**Figure 2**). Applying the same selection to both tests produced different results. Under shade, we selected the three pure lines and six F1 hybrids and under full sun, the same three pure lines and 10 F1 hybrids. Five of the hybrids were the same and five were different. In the full-sun selection scenario, the F1 hybrids (which inherited the dominant rust resistance genes from their maternal parent) produced 60–80% more than the best female line (CU1842). Regarding the final cupping score, four F1 hybrids had higher scores than the best maternal lineage (CU1842). In particular, the F1 hybrid "CU1842 × E047" which produced 40% more than the best female line, with a comparable tree volume and with a final score of over 85 and a bean size over 85%, appears to be exceptional.

Selection for Specialty Coffee

We used the Specialty Coffee Association's definition to define specialty coffee specifications. Through cupping, coffee tasters

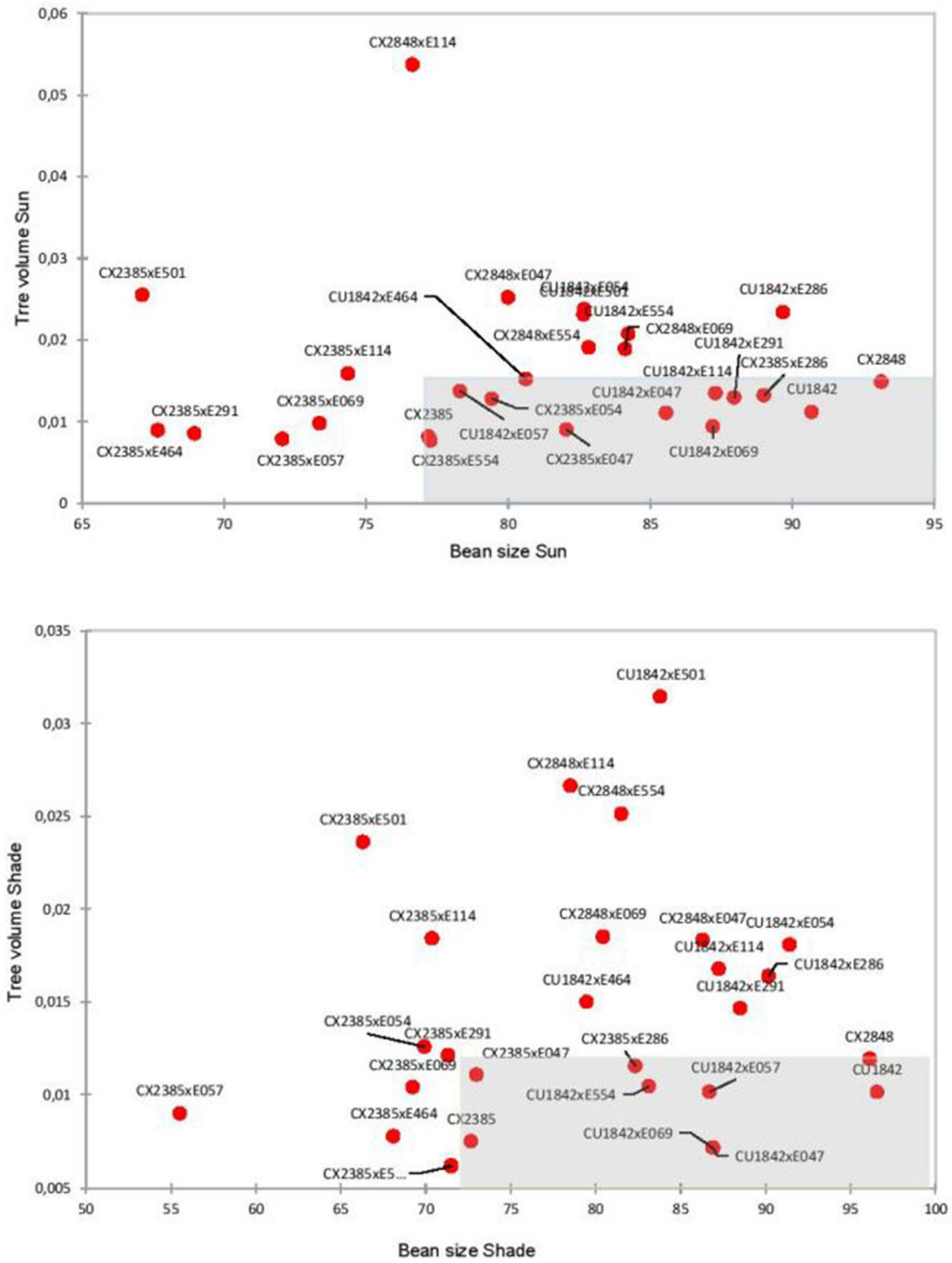

FIGURE 2 | Selection for a "full-sun" farming system. Scatterplots showing tree volume and bean size. The F1 hybrid population and the population of female lines (three components of cv. Castillo) are represented. The genotypes inside the rectangle were selected because they produced similar performances in terms of tree volume and bean size to those of the maternal lines. Upper graph, selection in the full-sun plot, lower graph, in the shade plot.

assess a coffee score and determine whether it is a specialty grade quality. For breeders, this means tracking down genotypes that produce the highest cup quality scores. In this definition, all the other traits are secondary. However, we considered that productivity is also an important criterion for farmers, so we included it in the two figures that deal with the selection of specialty coffee candidate varieties.

It is known that coffee quality is closely linked to environmental conditions and particularly to elevation (Bertrand et al., 2012). In the present study, selection was made at an elevation that could not generate very high scores (i.e., over 90), and we only selected genotypes with a final cupping score of over 85. Applying the same selection in full sun and under shade, produced quite different results. Three Ethiopian genotypes (E501, E286, E057) and three F1 hybrids stood out when selected in the sun trial (**Figure 3A**). These three F1 hybrids all had the same mother (CU1842). The productivity of the Ethiopian E286 was of the same order of magnitude as that of the F1 hybrids. The E286 genotype produces more than 4.5 kg of cherries per tree. This "wild" genotype can therefore produce a lot of very good coffee in full sun conditions. However, productivity has to be related to hectares. The tree volume was 3–4-fold higher. In practice, this would mean that the planting density would have to be reduced twofold (1,800–2,500 trees/ha for E286 compared to 4,000–5,000 trees/ha for the CU1842 × E047 F1 hybrid). One would thus expect half the productivity per hectare compared to the best hybrids (respectively, 2,000 kg vs. 4,800 kg of green coffee).

In the shade plot, only five Ethiopian genotypes were selected (**Figure 3B**). The E047 genotype produces more than 3.5 kg of cherries per tree. This "wild" genotype can therefore simultaneously produce a lot of coffee and very good coffee under shady conditions. Again, it is important to link this productivity to hectares. One would expect half the productivity per hectare compared to the best hybrids (respectively, 1,600 kg vs. 3,200 kg of green coffee).

Selection for "AFS"

There are no recognized specifications for this farming system. We believe it would be important to select the most productive genotypes, with large bean sizes, the best sensory scores and volumes compatible with crop densities that would allow high yields per hectare. Farmers who grow coffee in AFS are in competition with growers who cultivate coffee in full-sun conditions because coffee purchase prices do not take agroforestry into account. We thus set the yield threshold at the yield level of the best Castillo lineage grown in full sun, i.e., genotypes selected in the shade plot would have to produce at least as much as the best Castillo pure line grown in the full sun plot. For the final cupping score, as the best Castillo lineage achieved a final cupping score of 83.42 under shade, we set the threshold at 83. Regarding the seed size, the performance had to be at least that of the worst Castillo line. Regarding tree volume, we set a tree volume under shade that could not be more than 40% bigger than that of the largest female in full sun.

When this selection was applied, we retained two F1 hybrids whose characteristics are listed in **Table 2**. In shade conditions, the two selected hybrids (CX2385 × E054 and CU1842 × E286) produced, respectively, 22 and 2% more than the best pure line in full sun conditions. Both hybrids have a cup quality similar to that of the pure line. The size of the beans produced by one of the hybrids is smaller than the pure line while the size of the beans produced by the other is comparable with the pure line.

Clustering Genotypes for Further Selection in Suitable Farming Systems

At this early stage of preselection, the breeder can consider that the aim is to cluster tested genotypes rather than select them, which may be useful to subsequently dispatch the groups to coffee breeding hubs and/or to multi-location variety trials to test genotype x environment interactions. Genotypes may be selected across all traits by pooling them in groups based on common traits. This helps avoid an *a priori* bias. We tested this approach in both the sun and shade trials. This enabled us to determine if it would be useful to practice two types of pre-selection (under shade or in full sun) at this stage of selection. Clustering was based on yield, bean size, tree volume and the NIRS signature. The NIRS signature can be considered a preferable to costly sensory analysis at this stage of selection and non-selection context. To reduce the NIRS signature at one variable, we used the scores of the genotypes for the main first factor resulting from a discriminant analysis of the 15 main PCs.

We obtained three groups in the two plots (**Figure 4**), a group with male parents, another with the majority of the hybrids, and a final group with the Castillo pure lines and some hybrids. The male parents grouped together both under shade and in full sun. This cluster remained stable in any environment. The "F1 hybrid cluster" and the "pure line cluster" differed substantially under shade and in full sun conditions.

The majority of hybrids (16) formed the same group under both conditions, indicating comparable performance in terms of productivity, seed size, tree volume and biochemical composition. However, in full sun conditions, seven hybrids grouped with the Castillo lines while four hybrids grouped with the Castillo lines in the shade. Only three hybrids (CX2385 x E464, CU1842 × E554 and CX2385 × E554) clustered with the Castillo lines in both conditions, suggesting that the performances in terms of yield, bean size and biochemical composition of those F1 hybrids are close to the maternal pure line whatever the environment. Five hybrids changed clusters depending on the environment. These genotypes are therefore particularly adapted to one or the other of the two environments.

DISCUSSION

Evidence of the effects of increasing atmospheric levels of greenhouse gases has become clearer and more convincing (IPCC, 2013, 2014). After very alarmist studies that predicted drastic drops in coffee production (Bunn et al., 2015; Ovalle-Rivera et al., 2015), a recent study claims that coffee crops could survive climate change and global warming to a greater extent than previously estimated (DaMatta et al., 2019). It is therefore

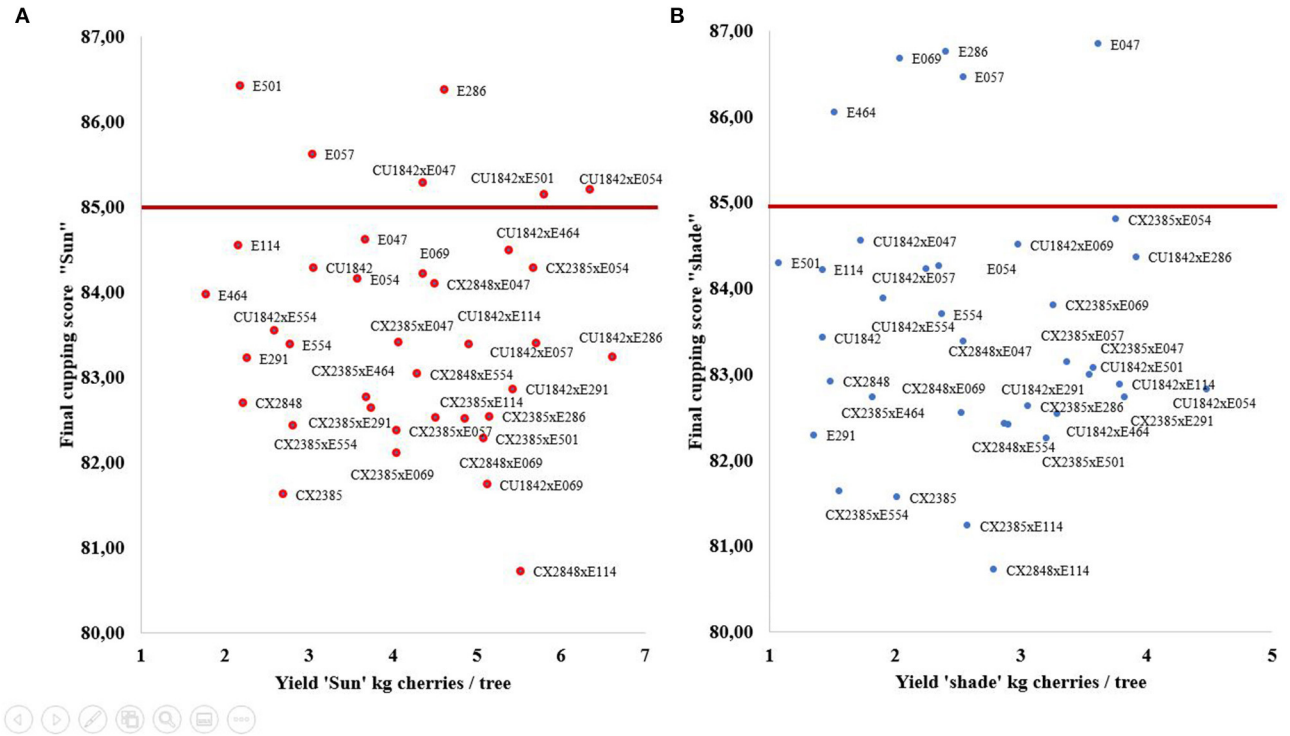

FIGURE 3 | Selection for "specialty coffee." Scatterplots showing the final cupping score and yield. The genotypes inside the rectangle were selected because they produced higher performances than the threshold (blue line) in terms of the final cupping score. **(A)** selection in the full-sun trial; **(B)** in the "shade" trial.

TABLE 2 | Performances in the shade plot of hybrids selected under shade, compared to the performances of the best maternal pure lines in full sun.

Genotype	Yield (kg of fresh berries per plant)	Final cupping score	Bean size (percentage bean size 16 to 20)	Recommended crop density (number of tree/ha)	Productivity/ha Kg green coffee
F1 hybrid CX2385xE054	3.76	84.79	69.93 *	4,500	3,384
F1 hybrid CU1842xE286	3.92 *	84.35	90.18	3,600	2,822
Pure line CU1842	*3.06*	*84.28*	*90.00*	4,500	2,754

Comparison of the target traits. Numbers in italics indicate the selection threshold. Numbers in red indicate the performances obtained in the shade trial. Numbers in black indicate performances obtained in the sun trial.
Means followed by an asterisk indicate a difference between the pure line CU1842 and the F1 hybrid according to the non-parametric Wilcoxon rank-sum test.

very difficult to predict the genotypes that should be used to best adapt to climate change.

For the selection of varieties adapted to climate change and global warming, the solution is undoubtedly to select genotypes for a wide range of scenarios at the same time by testing future varieties in as many environments as possible (i.e., by favoring genotype x environment studies). However, in tree breeding, in the first stage, genotypes are tested in a single environment to avoid increasing costs. The risk is to bias this first stage by selecting genotypes adapted to this particular environment. Here we chose to carry out the first stage of the selection in full sun and using artificial shading to mimic one of the main effects of AFS systems.

The Performances of the Cultivated and "Wild" Accessions Differed Markedly

We distinguished the following genotypes according to their origin and mode of reproduction. It was extremely interesting to compare the group of so-called wild Ethiopian genotypes with the three cultivated lines selected in Colombia. The three latter lines are clearly representative of the breeding initiatives that have been underway in Latin America for more than 50 years in response to two imperatives, reduce the size of the plant to enable a higher density per hectare and to select varieties that are resistant to orange rust.

The average tree volume of the lines from Colombia was 4- to 5-fold smaller than the original accessions from Ethiopia,

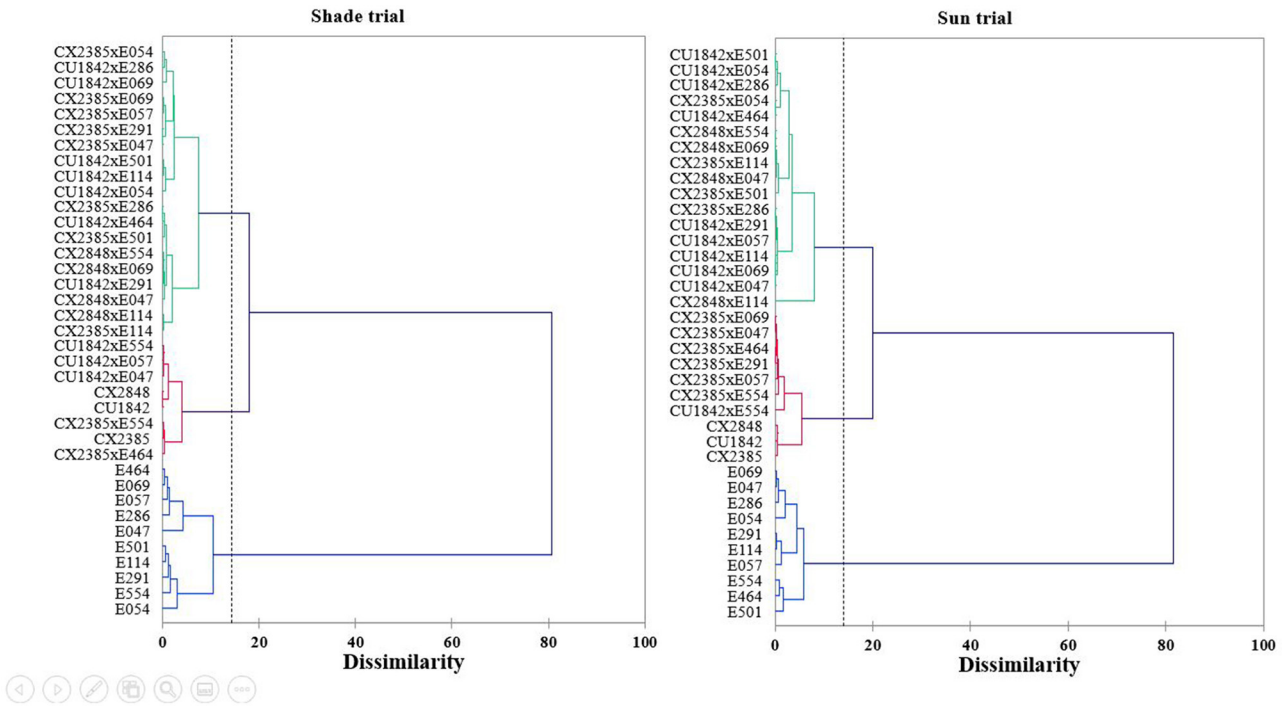

FIGURE 4 | Hierarchical cluster analysis (Euclidean distance, Ward's grouping method) resulting from the discriminant analysis of the 38 varieties studied based on yield, bean size, tree volume and NIRS signature, under shade and in full sun conditions. The upper graph corresponds to shade conditions and the lower graph to full-sun conditions.

illustrating the effectiveness of selection efforts. This reduction in tree volume enabled cultivation densities of 4,500 to 6,000 trees/ha compared to only 2,500 to 3,000 for the Ethiopian accessions. The reduction in plant volume is one of the pillars of the Green Revolution in cereals (Elias et al., 2012). Arabica coffee is no exception to this rule. In Arabica, the reduction in volume is based on a single gene that confers dwarfism. Dwarfism has also had substantial benefits in fruit tree production, enabling higher yields, and facilitating harvesting in orchards (Battistini and Battistini, 2005). In the case of Arabica, productivity per plant has not been increased as the productivity of the Castillo pure line was lower than that of the Ethiopian, both under shade and in full sun conditions. It is therefore productivity per hectare that has increased simply because of plant dwarfism, thereby confirming the findings of Moncada et al. (2019), who suggest that the yield potential of various Ethiopian accessions exceeds that of any pure line variety cultivated in Colombia today.

Regarding quality, the Castillo lines had large beans, which is a characteristic sought after by coffee buyers, whereas the Ethiopian accessions had much smaller bean sizes that are not compatible with the usual commercial standards. However, many Ethiopians accessions do have large beans. It turns out that the selection of the Ethiopian accessions used in this study did not take this criterion into account. In comparison, the homozygous lines of Castillo have very large seeds. In fact, selection on this trait can be highly effective, as its maternal heritability is high.

Ethiopian accessions in our study produce small beans but remarkable sensory quality. Indeed we provide evidence that bean size is not correlated with sensory quality, rather, it is the weight of 100 healthy green beans (g) that is correlated with sensorial quality (Marie et al., 2020).

On the other hand, the sensory quality of the Castillo pure lines appeared to be quite good: one of the lines (CU1842) had a final score over 84 both in full sun and under shade. There was no mention of off-type flavors during the tasting (data not shown), which confirmed that breeders have been able to circumvent the negative effects of introgression of chromosome fragments that have been reported in some Arabica varieties derived from the Timor hybrid (Bertrand et al., 2003). However, we noted that the selection resulted in a sensory quality inferior to that of most of the Ethiopian accessions. Commercial varieties produced and released in Colombia to date have been selected mainly for their high yield and resistance to leaf rust, while cup quality has been evaluated by discarding progenies whose cup quality differs from that of Typica, Bourbon and Caturra. There is therefore considerable room for improvement of this agronomic trait.

The Performances of the F1 Hybrids Are Far Beyond Exceed the Productivity of Both Parents

The performance of the F1 hybrids was remarkable in terms of productivity. They were much superior to the Ethiopian parents or cultivated lines, both in full sun and under shade. This confirmed our previous findings (Bertrand et al., 2011; Marie et al., 2020) in studies carried out using a factorial design or

in farmers' plots. The very high productivity of the F1 hybrids is due to the heterosis between the two parental populations (Bertrand et al., 2005). Although Arabica is a self-pollinated organism, a 30–80% heterosis level seems particularly high for an autogamous species, especially since a recent study (Scalabrin et al., 2020) showed that this species was responsible for upsetting a very recent allo-polyploidization event on the evolutionary scale (10,000 to 20,000 years ago), and since this species has been found to have extremely low levels of variation as a consequence of the allopolyploidization event. Unsurprisingly, it was not possible to predict the heterosis by the value of the average parent. The heritability of the yield, estimated in both environments, was close to zero. The genetic and molecular basis of heterosis remains elusive (Schnable and Springer, 2013). For a species as little polymorphic as the Arabica, our hypothesis favors complementarity of the parents in the regulation systems.

Bean Size and Sensory Quality Are Maternal-Like for Other Traits

The bean size of the F1 hybrids was close to that of the maternal lines. We observed a strong maternal effect for this trait in both full sun and shade conditions. The F1 hybrids generally inherited the best characteristics of the Castillo maternal lines. The seed size could be quite accurately predicted under shade based on the value of the maternal genotype. Finally, the tree volume of F1 hybrids was also close to that of the maternal lines. The dwarfism gene in cv. Caturra was dominant and was expressed in all the F1 hybrids.

In terms of sensory quality, the results obtained for the F1 hybrids were quite close to those of the maternal lines, but with greater variability, suggesting that this trait has selection potential. This is consistent with the findings of a previous study (Bertrand et al., 2006). There was no apparent heterosis for this character, but it would be hard to predict it based on the average value of both parents and heritability was not significant. The fact that the F1 hybrids were closer to the maternal lines than to the Ethiopian accessions suggests that, like bean size, maternal heritability was strong for this trait. However, the fact that the hybrids grouped between the two parental groups based on the NIRS signatures strongly suggests that biochemical composition is predominantly additive (**Supplementary Figure 2**). We now need to confirm whether the direction of the cross has a strong impact on sensory quality.

Partial Rust Tolerance

Castillo varieties that have been specially bred for rust resistance were found to have a higher incidence of the disease than accessions from Ethiopia. Ethiopian accessions carry resistance genes (SH1, SH2, SH4, SH5) that are all overcome by the rust strains found in Colombia. Colombia lineages (including cv. Castillo) derived from the Timor hybrid, which bears SH6 to SH9 genes, are expected to have incidence levels close to zero. The levels achieved here showed that the fungus was able to overcome the resistance genes SH6 to SH9 in all three lines. In fact, several varieties developed in Brazil using different sources of rust resistance are today completely susceptible or only partially resistant (Zambolim, 2016). The situation is the same throughout Latin America where the rust races have overcome resistance genes deployed in the varieties derived from the Timor hybrid. This is becoming a major concern in the coffee sector. In this study, we were not surprised to find that the vertical resistance of the three lineages of Castillo has been overcome. It is known that once this so-called vertical resistance is overcome, a good dose of partial resistance may remain. According to Alvarado-Alvarado et al. (2005b), over 80% of the genotypes involved in the Castillo variety have incomplete resistance and the three lines chosen for our study shared this feature. Surprisingly, we found that this partial resistance was lower in the Castillo lines than in the Ethiopian accessions and in the F1 hybrids.

How should we interpret the greater tolerance of Ethiopian parents and F1 hybrids to rust compared to homozygous lines? Is it due to persistent heterozygosity in the Ethiopians and *a fortiori* in the F1 hybrids? Is it due to a better leaf/fruit balance, as observed by Echeverria-Beirute et al. (2018) and or better plant health ("good plant health," a concept revisited by Döring et al. (2012). We developed this health concept in a recent work (Toniutti et al., 2017; Echeverria-Beirute et al., 2018). Indeed, the incidence of rust in susceptible varieties increases with the fruit load relative to total tree biomass (Avelino et al., 2006; Echeverria-Beirute et al., 2018). This suggests that breeding of homozygous lines for productivity and dwarfism could lead to the creation of varieties with reduced plant health.

Comparison of Performances Under Shade and Full-Sun Conditions

The shade imposed on the plants in our study was homogeneous and strong. The coffee yield was reduced by 40% when more than 55% of the natural light was filtered out. In a study with a similar design in which 45% of the natural light was filtered out, shade was reported to reduce coffee tree productivity by 18% (Vaast et al., 2006). Jaramillo-Botero et al. (2010) found that trees under 48% shading yielded 20% less than trees grown in full-sun conditions. In Brazil, coffee productivity was found to be reduced by 15% in coffee plantations of *Grevillea robusta* trees grown under shade (Baggio et al., 1997). In natural conditions, full-sun coffee production was 30% higher than that of shaded coffee in Costa Rica, while in Nicaragua full-sun coffee production was equal to that of shaded coffee (Haggar et al., 2011). *Coffea arabica* is an understorey plant in its area of origin. By nature, this species tolerates high levels of shade and shade cannot be considered as a suboptimal environment. However, in the present study, the impact of shading on production levels was high probably because our artificial shading was both strong and uninterrupted.

Bean size and final cupping score seemed to be? was? relatively unaffected by artificial shading. There were disparities between genotypes, suggesting there is room for selection. The accessions from Ethiopia were the most sensitive to light regimes. This result was out of line with what has been observed in other studies where shade positively affected bean size as well as beverage quality (Vaast et al., 2006). This difference could stem from the fact that Vaast et al. only worked on one genotype.

Bean Size and NIRS Signature Are Stable Characters While Yield and Above All Sensorial Scores Are Less Stable

We observed high stability in the genotype classifications of bean size, meaning selection for bean size would produce the same results if carried out under shade or in full-sun conditions. The fact that shading is known to reduce the ambient temperature (Ehrenbergerová et al., 2017; Da Silva et al., 2018) by about 4°C at the leaf surface implies that bean size selection is not sensitive to shading or temperature. Using the NIRS signature showed revealed high stability in the distances between varieties regardless of the extent of shading, which is consistent with our previous findings (Posada et al., 2009). We found a correlation between the yields of genotypes grown in the shade and in the sun, but the correlation was not strong enough to predict the productivity of a genotype in the shade by knowing its performance in full sun (and vice versa). Selection for green coffee yield therefore depends on light intensity and/or temperature. We found no correlation between genotype ranking and the final cupping score between the two environments. Unfortunately, this is a time-consuming and expensive method, especially as it involved a large number of samples at this pre-selection stage. If we assume that the biochemical composition of the grain reflects its sensory quality, as we observed high stability using the NIRS, we can hypothesize that the absence of a correlation between final cupping score in the two environments was due to the lack of precision inherent to the sensorial test. However, NIRS measures the biochemical composition of the grain, i.e., the aroma precursors, not volatile compounds. Two future lines of research will therefore be conducted and the findings compared to those obtained using the sensorial method. First, it would be interesting to test whether a NIRS signature can advantageously replace sensory measurements. In this system, the distance from well-known varieties will be evaluated. Selection will be based on the candidate's distance from well-known varieties. When the distance is short, this indicates that the varieties are close to each other and are therefore considered to belong to the same "sensorial universe." In a second experiment, it could also be considered that differences between Arabica genotypes are mainly due to volatile compounds. In this case, we would routinely use SPME-GC-MS analysis to classify the genotypes in different groups based on their volatile compounds. We would then a analyse the convergence of the three methods (i.e., using classical sensory analysis, NIRS and SPME-GC-MS) and choose the most repeatable and cheapest method.

Does Selection in Full Sun or Under Shade Lead to the Same Results? The Answer Depends on the Specifications

Specifications dictated objectively or subjectively by the market, producers and/or political authorities are the result of implicit or explicit choices based on rational or irrational interests. Breeders try to fully respect the stakeholders' specifications.

In the present study, we opted to illustrate selection specifications corresponding to three main farming systems which produce coffees for three types of markets corresponding to three "breeding orientations" as defined by Lammerts van Bueren et al. (2018). We showed that the selection of genotypes according to specialty coffee specifications resulted in the selection of coffees only of Ethiopian origin that were remarkable for their sensory quality under shade conditions, and three Ethiopian accessions and three F1 hybrids under full-sun conditions. In this "corporate-based breeding" approach, it is clear that selection in full sun does not produce the same results as in the shade. We therefore strongly recommended from the first stage of selection on, to test the genotypes both under shade and in full sun. On the other hand, for the sensory qualities to be fully expressed, we recommend carrying out this selection at a high altitude. The Ethiopian accessions tested in this study were collected at high altitude in Ethiopia. They are therefore suitable for cultivation at high altitudes, and this is where they will best express their sensory qualities. It seems that there could be a specific adaptation of some of these genotypes to shade or on the contrary, to full light at high altitudes (see for example, the remarkable performance of the E286 genotype in full sun compared to its poor performance under shade). It would be interesting to test for this specific adaptation in several dozen genotypes. The Ethiopian accessions, on the other hand, are rarely adapted to low altitude conditions with higher temperatures (personal observations). One defect we identified is their small bean size. By selecting on a wider genetic basis, it will probably be possible to find Ethiopian accessions of excellent quality with large beans. It would also be useful to breed 100% wild Ethiopian varieties by crossing accessions from Ethiopia to obtain F1 hybrids that are more productive than the Ethiopian accessions and have an excellent sensorial profile. This work has already been done in Ethiopia itself (Opile and Agwanda, 1993; Bellachew, 1997) but unfortunately the material is not yet available for other producing countries.

The main problem in selecting Ethiopian lines or Ethiopian F1 hybrids concerns tree volume. The volumes of the selected trees vary 2- to 4-fold compared to the Castillo lines or F1 hybrids. This means that the crop densities and consequently yields per hectare are reduced accordingly. This would be acceptable for farmers if the coffee were to be sold at 1.5 or twice the coffee price set by the New York Stock Exchange to compensate for the producers' loss of income. The specialty coffee market needs to consider this limitation in order to continue to secure sustainable supplies of specialty coffee.

A very large part of the current world coffee orchard is composed of dwarf varieties (small tree volume) well-adapted to full sun (i.e., Catuai, Caturra, IAPAR59, CR95, Castillo®, Marsellesa®, Obata, etc.), with mainstream sensory quality and large beans for the pure lines that make up the Castillo multi-line variety. Our starting point was the observation that selection for full sun must first focus on the volume of the plant to maximize production per hectare. We have shown that it is much better to use F1 hybrids, which produce considerably more with the same plant volume. These hybrids also had the same sensory and physical qualities as the maternal lines. In a recent article (Marie et al., 2020), we showed that two F1

hybrids (Centroamericano-H1 and Mundo Maya-H16) with a volume only 20% higher than that of Marsellesa® or Caturra (one of the most widely cultivated varieties) produced 50% higher yields than Marsellesa® or Caturra. In the present study, 10 F1 hybrids produced 60–85% more than the best female line (CU1842) with the same tree volume (i.e., resulting in the same cultivation density). The results of this study confirm previous reports: substantial genetic progress can be expected from the dissemination of F1 hybrids (obtained by crossing American dwarf x wild Ethiopian varieties) in Arabica farming systems based on the principles of the first Green Revolution (full-sun dwarf varieties, mainstream cup quality). The question of whether during selection, the trees should also be tested for their ability to produce under shade does not arise.

In the third type of selection, we opted to select varieties for their adaptation to shade conditions. Indeed, even in Brazil (the world's leading coffee-producing country) where cultivation under full sun largely predominates, some voices which suggest that AFS may represent a better management system for biodiversity and soil conservation are finally being heard. The main shortcoming of AFS is lower productivity compared with the intensive full-sun system. In a previous multi-location trial in an agroforestry system (Bertrand et al., 2011), hybrid yields were shown to be 58% higher than those of the American cultivars. In the present study, we have shown that F1 hybrid varieties are far more productive than others under shade. It is therefore possible to select hybrids that produce in the shade as well as the best pure line varieties grown in full sun. This finding is a powerful argument to encourage coffee growers who use the full-sun cropping system to change to AFS cropping systems, especially in regions where temperatures are expected to increase in climate scenarios. For the selection of genotypes adapted to agroforestry systems, we advise proceeding as we did in this study (i.e., selecting the genotypes that produce as much under shade as the best controls in full sun). For this farming system to be profitable, the productivity levels indeed need to approach those of farming systems in full sun. However, this advantage will disappear the day growers in full-sun adopt F1 hybrids. To really progress, it will be necessary to select hybrids which produce as much in full sun as under heavy shade. **Data sheet 1** shows that this is the case for the hybrid CX2385 × E291. Indeed, although the volume of the tree is not modified by shading and the number of nodes formed does not change either (data not shown), the number of flower buds does. How does light modify the number of flower buds? Is it the quantity or quality of light that has an effect? Are there any allelic variations for this trait? At a time when global warming is accelerating and agroforestry is perceived as a mitigation solution, it is urgent to launch research on this subject.

In both conditions (full-sun or shade), by opting for F1 hybrids rather than Ethiopian accessions it would be possible to increase crop densities and productivity per hectare while at the same time achieving good sensory qualities. However, being unable to achieve the quality of the Ethiopian varieties under shade and the fact of selecting only three hybrids under full-sun conditions is a matter of concern. Selecting a genotype with both remarkable sensory quality and F1 hybrid productivity per hectare appears to be exceptional? unlikely? for the time being.

The results we obtained also raise questions concerning the type of varieties. The first question concerns maternal heritability of cup quality. Should Ethiopian accessions be used as maternal parents? Indeed, the majority of F1 hybrids created in Latin America up to now were bred with an American pure line as mother and an Ethiopian accession as father. The heritability of good cup quality is perhaps mainly maternal, as is the case with bean size.

Can we imagine creating exceptional pure lines that combine both the productivity of dwarf F1 hybrids and the quality of the best "wild" Ethiopian accessions? This raises the question of the heritability of characters and the nature of heterosis in Arabica. Can it be fixed in the homozygous state? The path is long and hazardous. The nature of heterosis in an allopolyploid plant is still very poorly understood (Solhaug et al., 2016) but we suspect that it not very "fixable." To create high performance lines for productivity and quality, a very broad genetic base is needed to have a chance of selecting the best candidates. This strategy is only used in Ethiopia, the primary centre of diversity of the species, since the genetic diversity available there is very high. In other countries which do not have access to very wide diversity, in other words, all the other countries in the world, this strategy will probably quickly reach its limits.

So, if the F1 hybrid is the best type of variety to adapt to different farming systems and to climate change, the main problem to be solved is that of seed reproduction (Etienne et al., 2018). We have shown that this obstacle can be overcome by using male sterility, which makes it possible to produce F1 hybrid seeds at a price compatible with high-density cultivation (Georget et al., 2019). The transfer of male sterility genes to good progenitors should now be a research priority for the Arabica breeder community.

CONCLUSION

Although in this study, we did explore the main farming systems, others remain to be explored. Coffee harvest mechanization in Brazil is based on tall varieties (Mundo-Novo, Bourbon) which were not covered in the present study. We believe that tall F1 hybrids could be tested advantageously in mechanized systems where they may perform better than Mundo Novo and Bourbon. In the future, we could also test a selection based on both tree volume and productivity. In our opinion, there is no doubt that hybrids between tall American varieties and Ethiopian accessions will be more productive than their own parents.

It is also up to agronomists to develop new farming systems, especially to address the Anthropocene crisis. We could imagine, for instance, designing ultra-intensified farming systems with super-dwarf plant formats. Indeed, dwarfism genes exist that are even more pronounced than the Caturra gene (i.e., Laurina). This would allow greenhouse cultivation and, in theory, the cultivation of Arabica coffees outside the intertropical zone (Djerrab et al., 2020) to escape global warming.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

BB is the writer of the paper. AV established the trial, organized the observations, and collected and interpreted the results. LM statistically analyzed the results. J-CB helped to revise the document and to interpret the results. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.709901/full#supplementary-material

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Combining Ability and Molecular Marker Approach Identified Genetic Resources to Improve Agronomic Performance in *Coffea arabica* Breeding

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Plant breeding aims to develop cultivars with good agronomic traits through gene recombination and elite genotype selection. To support *Coffea arabica* breeding programs and assist parent selection, molecular characterization, genetic diversity (GD) analyses, and circulating diallel studies were strategically integrated to develop new cultivars. Molecular markers were used to assess the GD of 76 candidate parents and verify the crossing of potential F_1 hybrids. Based on the complementary agronomic traits and genetic distance, eight elite parents were selected for circulating diallel analysis. The parents and 12 hybrids were evaluated based on 10 morpho-agronomic traits. For each trait, the effects of general and specific combining abilities, as well as the averages of the parents, hybrids, and predicted hybrids, were estimated. Crosses that maximize the genetic gains for the main agronomic traits of *C. arabica* were identified. Joint analysis of phenotypic and molecular data was used to estimate the correlation between molecular GD, phenotypic diversity (PD), phenotypic mean, and combining ability. The selection of parents that optimize the allele combination for the important traits of *C. arabica* is discussed in detail.

Keywords: coffee breeding program, microsatellite markers, genetic diversity, hybrid certification, diallel analyses

INTRODUCTION

Coffee breeding programs aim to develop cultivars with agronomic and technological traits demanded by producers, combined with high productive potential, adaptation to different producing regions, and better cup quality (De Paiva Barbosa et al., 2019a). However, the genetic gain obtained through *Coffea arabica* selection is limited, mainly due to its low genetic variability (Setotaw et al., 2013). The recent origin, preferentially autogamous reproduction, and limited dispersion of the species are the primary reasons for this narrow genetic basis (Merot-L'anthoene et al., 2019; Scalabrin et al., 2020). Thus, efficient strategies to explore genetic variability are crucial for the parent selection and the success of *C. arabica* breeding programs (Alkimim et al., 2017). An

alternative would be to estimate and explore genetic variability through molecular markers (Sousa et al., 2019; Alkimim et al., 2020).

Molecular markers are of great use for genetic improvement and selection because they allow precise access to information at the DNA level (Ferrão et al., 2015). In *C. arabica*, these markers have been used successfully for germplasm and cultivar fingerprinting, genetic diversity (GD), genetic mapping, and marker-assisted selection (Missio et al., 2011; Alkimim et al., 2017; Setotaw et al., 2020). Molecular markers also facilitate controlled crossbreeding certification in breeding programs. *Coffea arabica* is an autogamous species and hybridized artificially. In this process, selfing must be prevented using a secure sterility system (Longin et al., 2012). However, female coffee plants are emasculated near the flower opening when the stigma is ripe and pollination proceeds. Therefore, true hybrid assessment is difficult because self-fertilization may occur before out-of-crossing prevention. It provides inaccurate progenies that can adversely affect all stages of future breeding programs. These problems can be overcome with molecular marker assistance (Caballo et al., 2018; Chauhan et al., 2021). By analyzing the allelic profile of the parents, molecular markers quickly and accurately identify self-fertilized progenies, distinguishing them from hybrid progenies (Conceição et al., 2011; Stetter et al., 2016). This strategy is particularly important for breeding perennial and long-cycle species with low genetic variability, such as *C. arabica*.

Another strategy to explore GD among individuals and select genetic resource to be included in breeding programs is to use diallel crosses. Diallel analysis evaluates the general and specific combining abilities of the parents and predicts the average behavior of the hybrids (Kaushik and Dhaliwal, 2018; Maioli et al., 2020). This approach is widely used to identify elite parents for developing hybrids or cultivars for use in breeding programs, understand hybrid heterosis, and provide information about gene action (Moura et al., 2016; Pereira et al., 2017; Ofori and Padi, 2020; Olivo et al., 2020). For coffee, diallel study was carried out to compare the performance of parent lines and hybrids of *C. arabica* (Cilas et al., 1998) and *Coffea canephora* (Cilas et al., 2003, Cilas and Bouharmont, 2005) and the data was used to assist the Cameroon breeding program (Cilas et al., 1998). In addition to its usefulness in breeding, diallel data also has been used to study the heritability of physical and mechanical properties of coffee wood (Cilas et al., 2006).

Among the different methods proposed for diallel crossing, circulating diallel (Kempthorne and Curnow, 1961) aims to reduce the number of hybrids to be evaluated and predicts the best unused hybrids. Thus, it is an efficient design and requires little mating efforts and experimental resources for plant material evaluation. It is of particular interest in the breeding programs for commercially important tree species (Tello et al., 2019), such as coffee.

Both molecular markers and diallel crosses have been used in different crops to analyze the relationships between genetic distance, agronomic performance, heterosis, and combining ability of hybrids. The results have been contrasting depending on the crop, genotypes, markers, and traits evaluated (Kaushik et al., 2018). Combining ability and genetic distance is highly correlated when related genotypes are crossed, as observed in the same heterotic group of corn (Makumbi et al., 2011) and related sunflower strains (Reif et al., 2013). Since *C. arabica* has a very narrow genetic base (Setotaw et al., 2013; Sousa et al., 2017b), the association of information on GD using molecular markers and the average behavior of genotypes based on morpho-agronomic traits may be useful in selecting parents.

This study aimed to use different approaches to assist the selection of parents to be introduced in coffee breeding programs for the main agronomic traits. *Coffea arabica* resources were selected based on a circular diallel mating design, for evaluating and predicting the performance of progenies in breeding programs. Diallel analysis and molecular markers genotyping were coupled to ensure the efficiency of coffee selection.

MATERIALS AND METHODS

Genetic Material

For the GD study, 22 coffee genotypes were evaluated, corresponding to cultivars and elite accessions of *C. arabica* breeding programs developed in Brazil. More than one plant from each cultivar/access was analyzed, totaling 76 coffee plants (**Table 1**). Eight genotypes were selected based on the complementary agronomic traits (**Table 2**) and genetic distance. They were crossed according to the circulating diallel model (**Table 3**). The potential hybrids were evaluated with molecular markers for controlled crossbreeding certification, and the true hybrids were planted.

The parents and hybrids were kept in the experimental area of the Department of Plant Pathology of the Universidade Federal de Viçosa (DFP/UFV), Brazil, region located at 20°44′26″S latitude, 42°50′54″W longitude and 665 m altitude. The annual temperature, considering the years of 2013 to 2016, varies from 5.4 to 37.5°C, with annual mean temperature of 20.3°C (20.1°C in the last 30 years) and annual mean precipitation of 1,220.5 mm (1,289.0 in the last 30 years). The seedling (in bags), with three pair of leaves, were planted in January, 2013, following a randomized block design, containing 20 treatments, composed of 12 F_1 hybrids and eight parents, with four replications and three plants per plot. The plants were arranged at a spacing of 3.0 × 0.70 m.

Phenotypic Evaluations

The circulating diallel crosses were phenotypically analyzed by observing and quantifying 10 phenotypic traits (**Table 4**): vegetative vigor (Vig), sprout color (SC), ripening fruit color (RFC), yield (Y), maturation cycle (MC), maturation uniformity (MU), ripening fruit size (RFS), coffee leaf rust (*Hemileia vastatrix)* incidence (CLR), brown eye spot (*Cercospora coffeicola*) incidence (BES), and leaf miner infestation (LM). The evaluations were carried out in May 2016 (41 month after plantation) in the experimental area of the DFP/UFV.

DNA Extraction and Amplification With SSR Markers

The genomic DNA of each plant was extracted from young and fully expanded leaves using a previously proposed method

TABLE 1 | Coffee cultivars and accessions used in the genetic diversity analysis.

Cultivar/Accession	Plant code	Location
Paraíso MG H419-1	1–10	Experimental field (Epamig)—Três Pontas, MG[a]
Catíguá MG2	11–16	Experimental field (Epamig)—Três Pontas, MG
Oeiras MG 6851	17–22	Experimental field (Epamig)—Três Pontas, MG
Acauã Novo	23	Experimental field (Fundação Procafé)—Varginha, MG
Arara	24	Experimental field (Fundação Procafé)—Varginha, MG
H484-2-18-12	25	Experimental field (Epamig)—Três Pontas, MG
Siriema	26	Experimental field (Fundação Procafé)—Varginha, MG
UFV 311-63	27	Experimental field (Epamig)—Três Pontas, MG
Sarchimor MG 8840	28–33	Experimental field (Epamig)—Patrocínio, MG
MGS Paraíso 2	34–37	Experimental field (Epamig)—Patrocínio, MG
Catuaí Amarelo IAC62	38–44	Experimental field (Epamig)—Patrocínio, MG
Bourbon Amarelo MG0009	45–49	Experimental Field (Epamig)—Patrocínio, MG
Híbrido de Timor MG 0357	50–54	Experimental field (Epamig)—Patrocínio, MG
Ibairi IAC 4761	55	Farm pântano—Patos de Minas, MG
Tupi Ferrero	56	Farm pântano—Patos de Minas, MG
Acauã	57	Experimental field of DFT/UFV, MG[b]
IPR103	58	Experimental field of DFT/UFV, MG
Obatã	59–60	Farm Paraíso—Varginha, MG
Obatã Vigoroso	61	Farm Paraíso—Varginha, MG
Catuaí Vermelho IAC144	62–64	Experimental Field (Epamig)—Três Pontas, MG
Topázio MG 1190	65	Experimental Field (Epamig)—Três Pontas, MG
IAC125RN	66–76	Farm Pântano—Patos de Minas, MG

[a] *MG, state of Minas Gerais, Brazil.*
[b] *DFT/UFV, Department of Plant Pathology, Universidade Federal de Viçosa.*

(Diniz et al., 2005). The quality and quantity of DNA were evaluated using a NanoDrop 2000 spectrophotometer. DNA purity was analyzed using 12 SSR primers (**Table 5**). These markers can distinguish and show the unique molecular profile of the main Brazilian coffee cultivars (Sousa et al., 2017b), including those analyzed in our work. PCR amplification was performed in a 20 μl reaction mix containing 50 ng DNA, 1 U Taq DNA polymerase, 1× enzyme buffer, 1 mM $MgCl_2$, 150 μM each dNTP, and 0.1 μM each primer, the volume was adjusted with sterile milli-Q water, using a PTC-200 thermocycler (MJ Research) and Veriti (Applied Biosystems). The reaction conditions were: initial denaturation at 94°C for 2 min; 10 cycles at 94°C for 30 s, decreased by 1°C each cycle (from 66 to 57°C) for 30 s; and 72°C for 30 s, followed by another 30 cycles of denaturation at 94°C, annealing at 57°C, and extension at 72°C, with 30 s each step. The final extension was performed at 72°C for 8 min. The amplified DNA was separated by electrophoresis in a 6% denaturing polyacrylamide gel and visualized using silver nitrate staining, according to a previously described protocol (Brito et al., 2010).

Genetic Diversity Analyses

Molecular marker data were coded as codominant for the performance of GD analyses. The dendrograms were constructed according to the unweighted pair group method using arithmetic averages (UPGMA) methodology in the MEGA software (7.0) (Kumar et al., 2016). The scores of the genetic dissimilarity matrix were obtained by the arithmetic complement of the weighted index in the GENES software (Cruz, 2013). The genetic distance was calculated as follows:

$$D_{ii'} = 1 - (\frac{1}{2}\sum_{j=1}^{L} p_j c_j)$$

Where:

Dii': genetic distance between the accession pairs i and i';
c_j: number of common alleles between the accession pairs i and i;
$p_j = \frac{a_j}{A}$: weight associated with locus j, determined by:
a_j: total number of alleles of locus j;
A: total number of alleles studied;

$$\sum_{j=1}^{L} p_j = 1$$

Diallel Analysis

The individual variance was analyzed using the data of 10 traits evaluated in the parents and hybrids, according to the following model:

$$X_{(ij)k} = m + g_{(ij)} + b_k + \varepsilon_{ijk}$$

Where:

$X_{(ij)k}$ = phenotypic score of the k-th observation regarding the ij-th genotype in the k-th block
m = general average;
= effect of the ij-th genotype (parent, $i = j$, or hybrid, $i \neq j$);
b_k = fixed effect of the k-th block;
ε_{ijk} = experimental error.

The superiority of the hybrid in relation to the others and/or the parents was evaluated using the Tukey test, with a 5% probability.

The general combining ability (GCA) and specific combining ability (SCA) were estimated using the parent and hybrid data, according to a previously described model (Kempthorne and Curnow, 1961). This diallel analysis was performed according to the following statistical model:

$$Y_{ij} = \mu + g_i + g_j + s_{ij} + \varepsilon_{ij}$$

TABLE 2 | Parents used in the diallel crossing.

N°	Parents	Origin	Main Features/Traits
1	Paraíso MG H 419-1	Catuaí Amarelo IAC30 × Híbrido de Timor UFV445-46	Dwarf cultivar, green sprout (young leaves), yellow ripening fruit, intermediated maturation cycle, large ripening fruit, resistant to coffee leaf rust, and nematode (*Meloydogine exigua)*
2	Catiguá MG2	Catuaí Amarelo IAC86 × Híbrido de Timor UFV440-10	Good cup quality, low nutrient demand, brown and green sprout (young leaves), red ripening fruit, intermediate maturation cycle, medium ripening fruit, coffee leaf rust resistant
3	Oeiras MG 6851	Caturra Vermelho CIFC19/1 × Híbrido de Timor CIFC 832/1	Excellent plant architecture, large grains, uniform fruit maturation, brown sprout (young leaves), red ripening fruit, semi-late maturation cycle, large ripening fruit, partial coffee leaf rust resistant
4	H484-2-18-12	Mundo Novo IAC515-3 × Híbrido Timor UFV443-3	Elite genotype (F_3 generation) with high yield, high vegetative vigor, and resistant to coffee leaf rust
5	UFV 311-63	Caturra CIFC426/2 × S.333 CIFC 254/14	Elite genotype carrying the coffee leaf rust resistance gene S_H3
6	Arara	Obatã × Catuaí Amarelo	High yield, green sprout (young leaves), yellow ripening fruit, late maturation cycle, large ripening fruit, coffee leaf rust resistant
7	Acauã Novo	Mundo Novo IAC388-17 × Sarchimor IAC1668	Drought tolerant, green sprout (young leaves), red ripening fruit, semi-late maturation cycle, medium-size ripening fruit, coffee leaf rust resistant
8	Siriema	[(Blue Mountain *C. racemosa*) × Mundo Novo] × Catimor UFV417	Drought tolerant, green sprout (young leaves), yellow ripening fruit, early maturation cycle, medium-size ripening fruit, partial coffee leaf rust resistant, leaf miner resistant

Where:

Y_{ij} = mean score of the hybrid combination ij ($i \neq j$) or the i-th parent ($i = j$). $Y_{ij} = (1/r)\sum_{k=1}^{r} X_{(ij)k}$, where r is the number of repetitions;
u = overall means of hybrid combinations;
g_i = effects of GCA of the i-th parent;
g_j = effects of GCA of the j-th parent;
s_{ij} = effect of SCA; and
ε_{ij} = mean experimental error.

The potential of hybrid combinations not obtained in the diallel (as the model was circulating diallel) was predicted using the following equation:

$$\hat{Y}_{ij} = u + \hat{g}_i + \hat{g}_j$$

Where:

$\hat{Y}_{ij}$ = predicted score of the hybrid ij;
μ = general average;
$\hat{g}_i$ and $\hat{g}_j$ = estimates of general combining capabilities

To assess the existence of significant differences between the effects of GCA and SCA, confidence intervals with a 95% probability were calculated. In this procedure, the bootstrap approach was adopted with the establishment of 5,000 new data sets obtained from the resampling of the original data. These sets were again submitted to diallel analyzes, generating estimates of the combining ability. The new estimates were ordered and, from the set, 5% of the extreme values were excluded (2.5% at each end), allowing the identification of limit estimates representing the desired confidence intervals.

TABLE 3 | Crossings performed according to the circulating diallel model.

Hybrid code	Female parent	Male parent
H14	Paraíso MG H 419-1	H484-2-18-12
H15	Paraíso MG H 419-1	UFV 311-63
H16	Paraíso MG H 419-1	Arara
H25	Catiguá MG2	UFV 311-63
H26	Catiguá MG2	Arara
H27	Catiguá MG2	Acauã Novo
H36	Oeiras MG 6851	Arara
H37	Oeiras MG 6851	Acauã Novo
H38	Oeiras MG 6851	Siriema
H47	H484-2-18-12	Acauã Novo
H48	H484-2-18-12	Siriema
H58	UFV 311-63	Siriema

Correlation Analysis

Pearson's correlation coefficient among parental GD, parental phenotypic diversity (PD), parental SCA, and hybrid phenotypic mean was estimated. Genetic diversity was obtained using the genetic distance matrix of the parents and analyzed using SSR markers. Phenotypic diversity was calculated by comparing the mean scores of each trait evaluated in the parents. The SCA was estimated using diallel analysis and the phenotypic means using the mean scores of each phenotypic trait evaluated in the

TABLE 4 | Phenotypic traits evaluated in eight progenitors of arabica coffee and 12 F_1 hybrids obtained by crossing in a circulating diallel scheme.

Variable	Description
Vig	Vegetative vigor (score scale ranging from 1 to 10). 1, fully depleted plants (low vigor) and 10, plants with maximum vegetative vigor.
SC	Sprout color (young leaves) (score scale ranging from 1 to 4). 1, green; 2, light bronze; 3, bronze; 4, dark bronze.
RFC	Ripening fruit color (score scale ranging from 1 to 3). 1, Red; 2, Yellow; 3, Orange.
Y	Yield. Liters of fresh cherries harvested per plant.
MC	Maturation cycle (score scale ranging from 1 to 5). 1, early; 2, semi-early; 3, intermediate; 4, semi-late; 5, late cycle.
MU	Maturation uniformity (score scale ranging from 1 to 4). 1, uniform; 2, semi-uniform; 3, semi non-uniform; 4, non-uniform maturation.
RFS	Ripening fruit size (score scale ranging from 1 to 3). 1, small, 2, medium; 3, large fruits.
CLR	Coffee leaf rust incidence (score scale ranging from 1 to 5). 1, absence of pustules and hypersensitivity reactions; 2, few leaves with spore-free pustules ("flecks") and with hypersensitivity reactions; 3, few pustules per leaf with high spore production and poorly distributed; 4, average number of pustules per leaf, distributed in the plant with high spore production; 5, large number of pustules with high spore production and high defoliation of the plant. NOTE: Plants with score 1 or 2, resistant; 3–5, susceptible.
BES	Brown eye spot incidence (score scale ranging from 1 to 5). 1, no symptoms; 2–5, leaves with *C. coffeicola* (2, low incidence and 5, high incidence).
LM	Leaf miner infestation (score scale ranging from 1 to 5). 1, immune. leaves without any lesion; 2, leaves with few tapered lesions; 3, leaves with few and small lesions; 4, leaves with moderate infestation, typical lesions, and live larvae; 5, leaves with severe infestation, typical lesions and live larvae.

TABLE 5 | SSR primers used to amplify the genomic DNA.

N°	Name	bp[a]	Sequence[b]	Reference
1	CaEST-006	227	F: CAGAATTGTTGTGGAGGGAAC R: ATGAAGCCAAACCAGAGACA	Ferrão et al., 2015
2	CaEST-022	207	F: GCCATTTCACAATCTCACCTC R: AGACCCAGCAGACAACAACA	Ferrão et al., 2015
3	CaEST-029	199	F: AGGAGATGCCTGTGACGAAC R: GGACGGAAAGATTCTGGCTTT	Ferrão et al., 2015
4	CaEST-031	109	F: CGGAGCAAGTGAATTGAACAGA R: AAAGGGAAAGGAAGAAGGAG	Ferrão et al., 2015
5	CaEST-040	101	F: TGAGCTAACCAAGACCAGTTCC R: CAACAGGAAATCACCGCCTA	Ferrão et al., 2015
6	CaEST-045	259	F: GCATCCTACCGAGTACATACAA R: TCCATCAACAACAACCGAAG	Ferrão et al., 2015
7	CaEST-048	151	F: TGAGACAAGCTATGGAGGAGGA R: AACCAGATCAACAGGGTAGGG	Ferrão et al., 2015
8	CaEST-071	155	F: ATGGAGAGGAAGACGCAACA R: CCTTATTGAAGACGCCCAAA	Ferrão et al., 2015
9	CaEST-072	197	F: TTGCTTGCTCCGCATCCTAC R: ATCGCTTCCAAGAGGCTTTC	Ferrão et al., 2015
10	CaEST-089	152	F: GTGAACCTCCCTTTCCCTTG R: ACTGGTCTCTCGTCTGTGAA	Ferrão et al., 2015
11	SSR016	140	F: ACCCGAAAGAAAGAAGAACCAAG R: CCACACAACTCTCCTCATTC	Combes et al., 2000
12	SSR095	352	F: TAAGAAGCCACGTGACAAGTAAGG R: TATGGCCCTTCTCGCTTTAGTT	Moncada and McCouch, 2004

[a] *bp, size of the amplified fragment in base pairs.*
[b] *F, foward primer; R, reverse primer.*

hybrids. A network graph of the correlations was constructed as proposed previously (Rosado et al., 2017). The thickness of the lines represents the absolute score of the correlation. The width of the line was controlled by applying a cut-off score of 0.5, for easy graph visualization.

RESULTS

Genetic Diversity

Genetic diversity was analyzed based on the SSR marker data obtained by genotyping 76 *C. arabica* plants corresponding to 22 cultivars or elite accessions with potential use in the coffee breeding program. The markers were able to discriminate the cultivars and the molecular profiles revealed polymorphism within cultivars/accessions. Since the genotypes of the same cultivar/accession were segregated, two analyses were performed. The first analysis used 22 individuals, one for each cultivar/access, and presented the most frequent alleles for each SSR primer. This study aimed to verify the distance between cultivars and accessions. A total of 28 alleles were obtained from the selected plants. The number of alleles amplified by markers varied between two (CaEST-006, 029, 031, 071, 072, 045, 048, and SSR16) and three (CaEST-022, 040, 089, and SSR95), with an average of 2.3 alleles per locus.

The highest genetic distance estimates were observed between Catiguá MG2 and Acauã (0.750), Catiguá MG2 and Topázio MG 1190 (0.696), Catiguá MG2 and Arara (0.643), Catiguá MG2

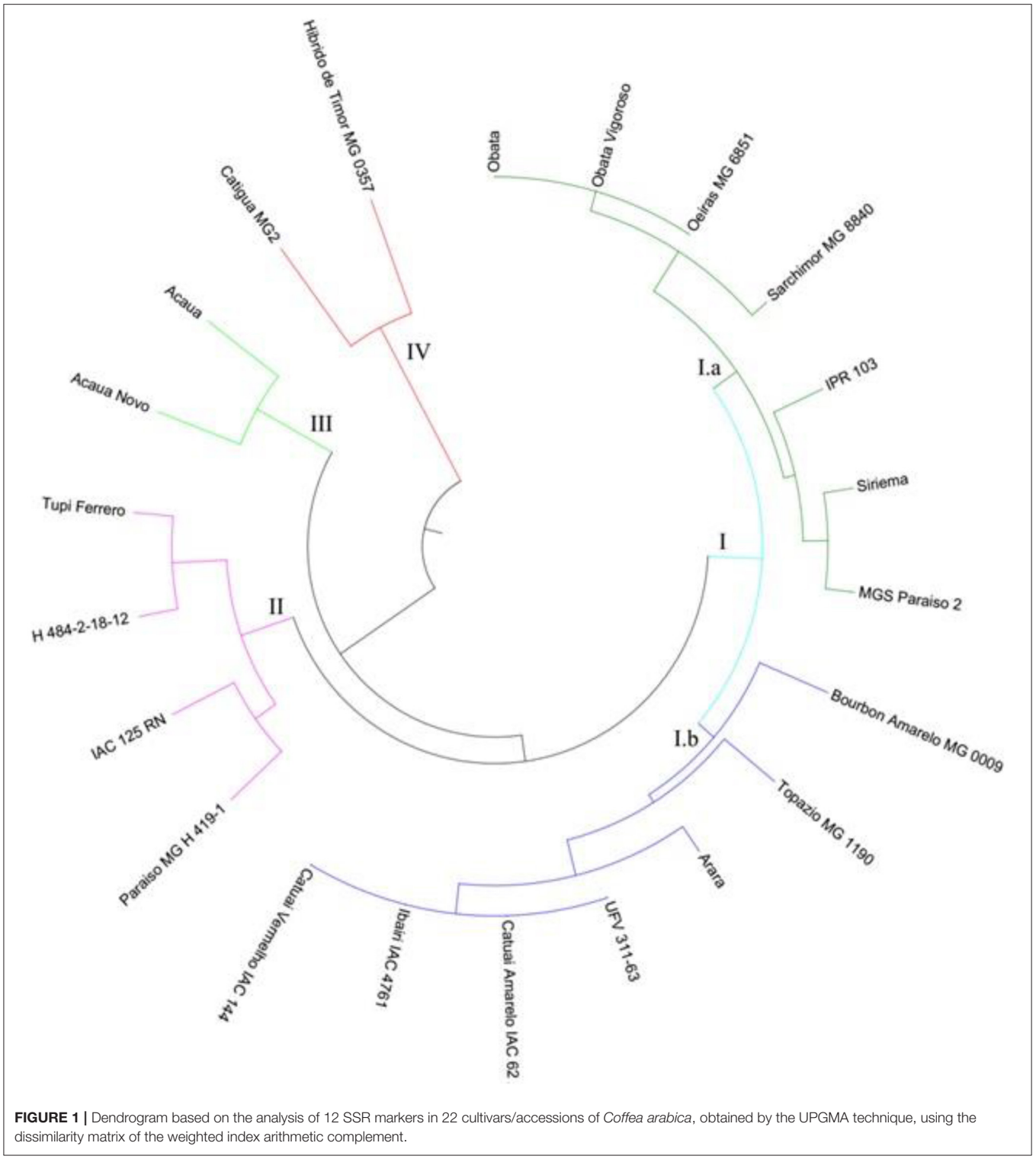


FIGURE 1 | Dendrogram based on the analysis of 12 SSR markers in 22 cultivars/accessions of *Coffea arabica*, obtained by the UPGMA technique, using the dissimilarity matrix of the weighted index arithmetic complement.

and Bourbon Amarelo MG 0009 (0.643), and Catiguá MG2 and Tupi Ferrero (0.643) (**Supplementary Table 1**). The clustering analysis of the 22 plants, based on the genetic distance matrix estimated between the pairs of individuals, using the 12 SSR markers, resulted in a dendrogram with four groups: I, II, III, and IV. Group I was subdivided into two subgroups: I.a and I.b (**Figure 1**).

A second GD analysis was performed considering the genotypic data of 76 *C. arabica* plants, including one or more plants per cultivar/accession. The clustering analysis, based

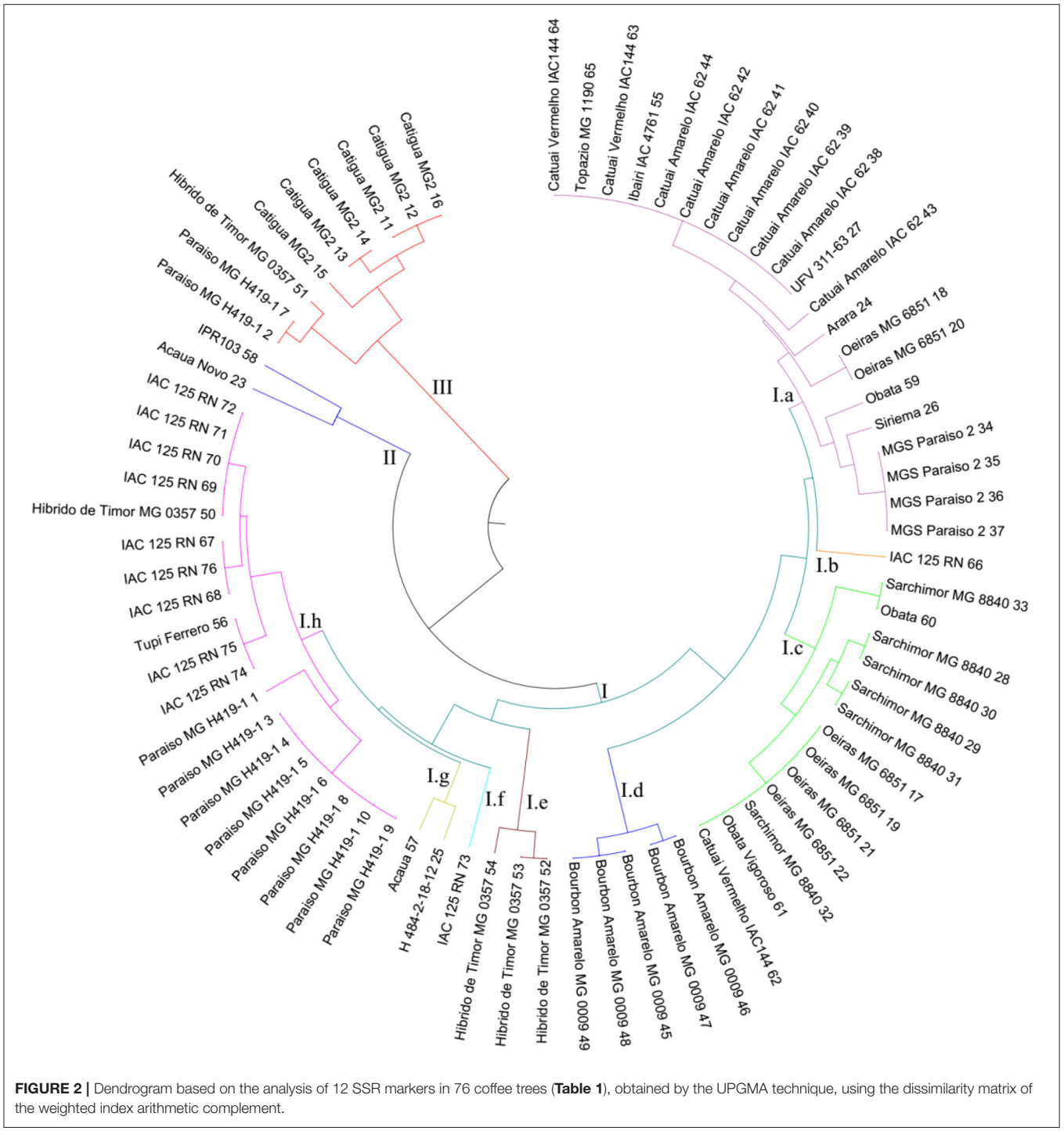


FIGURE 2 | Dendrogram based on the analysis of 12 SSR markers in 76 coffee trees (**Table 1**), obtained by the UPGMA technique, using the dissimilarity matrix of the weighted index arithmetic complement.

on the estimated genetic distance matrix between pairs of individuals, using 12 SSR markers, is shown in **Figure 2**. The dendrogram obtained consisted of three groups: groups I, II, and III. Group I was subdivided into eight subgroups: I.a, I.b, I.c, I.d, I.e, I.f, I.g, and I.h.

SSR marker analysis allowed us to observe polymorphisms within the nine cultivars/accessions: Catuaí Amarelo IAC 62, Sarchimor MG 8840, Oeiras MG 6851, Obatã IAC 1669-20, IAC 125 RN, Bourbon Amarelo MG 0009, Híbrido de Timor MG 0357, Catiguá MG2, and Paraíso MG H419-1. Some plants of the same cultivar/accession were allocated into different groups (plants of Paraíso MG H419-1, Híbrido de Timor MG 0357, Oeiras MG 6851, and IAC 125 RN cultivars). No polymorphism between individuals of Catuaí

TABLE 6 | Analysis of variance for 10 traits, evaluated in eight parents of arabica coffee and their hybrids obtained by a circulating diallel scheme.

		Mean square									
SV	**DF**	**VIG**	**SC**	**RFC**	**Y**	**MC**	**MU**	**RFS**	**CLR**	**BES**	**LM**
Treat	19	3.020**	0.098^{ns}	1.459**	5.168**	1.481**	0.379**	0.200^{ns}	0.150^{ns}	0.721**	0.134^{ns}
GCA	7	3.041**	0.099^{ns}	1.987**	5.802**	2.882**	0.614**	0.349^{ns}	0.313**	1.254**	0.061^{ns}
SCA	12	3.008**	0.097^{ns}	1.151**	4.797**	0.664**	0.241^{ns}	0.113^{ns}	0.056^{ns}	0.411^{ns}	0.177^{ns}
Error	57	0.542	0.071	0.267	1.669	0.332	0.200	0.167	0.114	0.299	0.125
Mean		6.652	1.191	1.865	3.743	3.396	2.842	2.217	1.840	2.733	1.235
CV(%)		11.064	22.303	27.735	34.516	16.960	15.740	18.422	18.371	19.990	28.678

SV, sources of variation; GSA, general combining ability; SCA, specific combining ability; CV, coefficient of variation; DF, degrees of freedom; Vig, vegetative vigor; SC, sprout color; RFC, ripening fruit color; Y, yield; MC, maturation cycle; MU, maturation uniformity; RFS, ripening fruit size; CLR, incidence of coffee leaf rust; BES, incidence of brown eye spot; LM, leaf miner infestation.

***significant by the F-test, at the level of 5%.*

^{ns}not significant.

Vermelho IAC 144 and MGS Paraíso 2 was observed (**Figure 2**).

Analysis of the Circulant Diallel

To assist the coffee breeding program, the most important *C. arabica* plants were selected for diallel analysis. The parental selection was based on the importance and complementarity of the agronomic traits (**Table 2**) and on the GD approach. The GD allowed the selection of coffee genotypes in all groups: Oeiras MG6851 and Siriema in group I.a; Arara and UFV 311-63 in group I.b; Paraíso MG H419-1 and H 484-2-18-12 in group II; Acauã Novo in group III; and Catiguá MG2 in group IV (**Figure 1**).

The potential F_1 hybrids developed, using eight cultivars/accessions crossed in a partial diallel, were evaluated with SSR markers to determine whether the progenies were obtained from controlled crossbreeding or self-fertilization. All parents were genotyped with SSR primers, and polymorphic and informative markers were identified for each hybrid progeny. Informative markers have polymorphisms between the parents; in this case, each parent must amplify at least one different allele. Thus, hybrid progenies have alleles present in both parents (**Supplementary Figure 1**), whereas self-fertilized progenies have alleles present only in the female parent (**Supplementary Figure 2**). Based on molecular markers, *C. arabica* plants confirmed as true hybrids and originated from the parent used in the artificial hybridizations were used in the diallel study.

The circulant diallel scheme estimates the genetic parameters and selects the best parents and hybrids based on the GCA and SCA scores. Using this partial diallel approach, a sample of possible crosses was studied, and the potential of all hybrid combinations was predicted. Therefore, this analysis provided information about the parents using few crosses without any information loss, since all hybrids were estimated in the model.

The variance analysis for the 10 evaluated traits, as well as the means of the effects for GCA and SCA, are presented in **Table 6**. The treatment effects were significant for Vig, RFC, Y, MC, Fruit MU, and BES traits, showing a variability among the genotypes (parents and hybrids). General combining ability effects were significant for Vig, RFC, Y, MC, MU, CLR, and BES traits. These estimates provide information on gene concentration (favorable allele frequency) with additive effects in the parents. Specific combining ability effect was significant only for Vig, RFC, Y, and MC traits.

The GCA scores were estimated for each parent and the two best scores for Vig, Y, MU, CLR, and BES (significant for GCA using analysis of variance) are highlighted in bold in **Table 7**. Low GCA scores are desired for CLR and BES traits, since the lowest score is associated with plants resistant to these diseases. The same was considered for MU, as the lowest score indicates the highest uniformity. The estimates of the SCA effects for each hybrid and parent are shown in **Table 8**, and the best cross combination for Vig and Y, the most significant traits, are highlighted. The reliability of the CGA and SCA estimate were assessed from the confidence interval limits (**Supplementary Table 2**). In this analysis, if the range does not include the zero value, the estimate is statistically non-null. In addition, the confidence interval information is useful to assess whether there is a significant difference between the estimates in the case where the confidence intervals do not overlap.

Catiguá MG2 × UFV 311-63 and Catiguá MG2 × Acauã Novo were the best identified crosses for Vig. In this analysis, not only the best SCA effect, but also the cross involving at least one parent with a high GCA, were considered. Although both GCA and SCA effects were significant for RFC and MC traits, they were not highlighted in the table, as fruit color and MC did not affect the preference for the developed cultivar, but the information must be available for the grower.

The mean performance of the obtained hybrids and the predicted mean scores of the hybrid not obtained in the partial diallel design were estimated for each trait (**Table 9**). The hybrids from crosses Catiguá MG2 × UFV 311-63 and Paraíso MG H419-1 × Arara showed the highest mean scores for Vig (8.085 and 7.750, respectively). Hybrids from Catiguá MG2 × Acauã Novo and Paraíso MG H419-1 × UFV 311-63 had the highest mean scores for the Y trait (5.250 and 4.960, respectively). Of the 10 evaluated traits, Vig, RFC, Y, MC, and CLR showed significant

TABLE 7 | Estimated values for the general combining ability (GCA) effects of eight arabica coffee parents, based on the means of t10 agronomic traits.

	Estimates of the GCA Values									
Parent[a]	**Vig[b]**	**SC**	**RFC**	**Y**	**MC**	**MU**	**RFS**	**CLR**	**BES**	**LM**
1	−0.409	0.000	0.215	−0.204	0.013	0.222	−0.155	−0.056	0.215	0.003
2	**0.494**	0.074	0.236	**0.581**	0.116	−0.052	−0.164	−0.046	−0.131	−0.004
3	−0.537	−0.015	−0.143	−0.334	−0.270	**−0.179**	0.076	0.243	0.302	−0.031
4	−0.088	0.045	0.004	−0.308	0.423	−0.041	−0.036	0.088	**−0.178**	−0.034
5	0.045	0.085	−0.241	−0.650	−0.363	**−0.241**	0.147	**−0.070**	**−0.268**	0.110
6	**0.229**	−0.085	0.327	0.350	0.369	0.020	0.141	**−0.072**	−0.056	−0.021
7	0.172	−0.056	−0.477	**0.603**	0.119	0.211	−0.054	−0.069	0.238	0.009
8	0.093	−0.046	0.079	−0.036	−0.407	0.060	0.045	−0.018	−0.122	−0.032

[a] 1, Paraíso MG H419-1; 2, Catiguá MG2; 3, Oeiras MG 6851; 4, H484-2-18-12; 5, UFV 311-63; 6, Arara; 7, Acauã Novo; 8, Siriema.

[b] Vig, vegetative vigor; SC, sprout color; RFC, ripening fruit color; Y, yield; MC, maturation cycle; MU, maturation uniformity; RFS, ripening fruit size; CLR, incidence of coffee leaf rust; BES, incidence of brown eye spot; LM, leaf miner infestation. The two best scores for Vig, Y, MU, CLR, and BES (significant for GCA using analysis of variance) are highlighted in bold.

TABLE 8 | Estimated of the effects of specific combining ability (SCA) of 12 hybrids (in parentheses) and 8 parents based on the means of 10 evaluated agronomic traits.

	Estimates of S.C.A effects									
Cross[a]	**Vig[b]**	**SC**	**RFC**	**Y**	**MC**	**MU**	**RFS**	**CLR**	**BES**	**LM**
1 × 1	−1.377	0.017	−0.295	−1.777	−0.214	−0.078	0.094	−0.020	−0.163	−0.116
2 × 2	−0.434	0.162	−0.337	−0.467	−0.420	−0.238	0.112	0.044	−0.097	−0.060
3 × 3	−0.579	0.004	−0.578	−0.657	−0.106	−0.235	−0.036	0.171	−0.001	−0.174
4 × 4	−0.434	−0.030	0.128	−0.822	−0.118	−0.260	0.022	−0.015	0.040	−0.167
5 × 5	−0.909	0.138	−0.383	−1.400	−0.505	−0.028	0.240	0.051	−0.029	−0.038
6 × 6	−0.778	−0.021	−0.518	−0.402	−0.384	0.037	0.000	0.139	0.293	−0.111
7 × 7	−0.371	0.046	0.214	−0.573	−0.259	−0.222	0.140	0.048	−0.002	−0.252
8 × 8	−0.213	−0.099	−0.190	−0.295	−0.164	0.038	−0.057	0.070	0.093	0.079
(1 × 4)	0.262	−0.153	0.249	0.871	−0.332	0.392	−0.026	0.129	0.397	0.046
(1 × 5)	1.214	−0.026	0.746	2.072	0.202	0.012	−0.041	0.037	0.405	−0.016
(1 × 6)	1.278	0.145	−0.406	0.612	0.550	−0.248	−0.121	−0.127	−0.477	0.201
(2 × 5)	**0.894**	−0.185	−0.025	0.909	0.684	0.286	−0.281	−0.139	−0.169	0.241
(2 × 6)	−0.126	−0.180	0.490	−0.299	−0.046	0.108	0.139	0.028	0.038	0.037
(2 × 7)	0.099	0.042	0.209	**0.324**	0.202	0.081	−0.081	0.025	0.324	−0.158
(3 × 6)	0.403	0.077	0.952	0.492	0.258	0.067	−0.019	−0.179	−0.147	−0.016
(3 × 7)	0.213	−0.037	−0.245	0.361	−0.080	0.293	−0.157	−0.097	0.227	0.372
(3 × 8)	0.542	−0.047	0.450	0.460	0.034	0.109	0.248	−0.066	−0.078	−0.008
(4 × 7)	0.431	−0.970	−0.391	0.462	0.397	0.070	−0.043	−0.024	−0.546	0.290
(4 × 8)	0.175	0.310	−0.115	0.311	0.170	0.057	0.024	−0.075	0.069	−0.002
(5 × 8)	−0.290	−0.065	0.045	−0.181	0.124	−0.243	−0.158	0.001	−0.178	−0.149

[a] 1, Paraíso MG H419-1; 2, Catiguá MG2; 3, Oeiras MG 6851; 4, H484-2-18-12; 5, UFV 311-63; 6, Arara; 7, Acauã Novo; 8, Siriema.

[b] Vig, vegetative vigor; SC, sprout color; RFC, ripening fruit color; Y, yield; MC, maturation cycle; MU, maturation uniformity; RFS, ripening fruit size; CLR, incidence of coffee leaf rust; BES, incidence of brown eye spot; LM, leaf miner infestation. The best crosses estimated based on the SCA, involving at least one parent with high GCA, for the main significant traits (Vig and Y) are highlighted in bold.

differences between the means of treatments, according to the Tukey test at the 5% probability level (**Table 10**).

To access the occurrence and degree of heterosis, graphs were drawn considering the mean of the parentals and hybrids based on seven traits (**Figure 3**). SC, RFC, and MC were not analyzed as these traits did not affect the cultivar preference. Higher levels of heterosis were found for Vig and Y. The data allow us to select hybrids that surpassed the average of their parents in some traits. Hybrid H15 showed high heterosis for Vig and Y, but low for other traits, including negative heterosis for RFS. H16 also showed high heterosis for Vig and Y and lower diseases incidence, but negative heterosis for MU and RFS. The performance of the hybrids is related to the combining ability of the parents for traits of interest. Thus, to reach heterosis for Y and Vig the best crosses are Paraíso MG H419-1 with UFV 311-63, H484-2-18-12, and Arara as well as Catiguá MG2 × UFV 311-63. However, the hybrids showed lower MU and RFS and higher incidence of BES and LM than one of

TABLE 9 | Hybrid means (in parentheses) and predicted mean values of the hybrid not included in the circulating diallel analysis for 10 agronomic traits.

Parent[a]	1	2	3	4	5	6	7	8
VIG[b]								
1	–	6.738	5.707	(6.418)	(7.503)	(**7.750**)	6.415	6.335
2		–	6.610	7.059	(**8.085**)	(7.250)	(7.418)	7.240
3			–	6.028	6.160	(6.748)	(6.500)	(6.750)
4				–	6.609	6.793	(7.168)	(6.833)
5					–	6.926	6.869	(6.500)
6						–	7.053	6.974
7							–	6.917
8								–
SC								
1	–	1.264	1.176	(1.083)	(1.250)	(1.250)	1.134	1.145
2		–	1.250	1.309	(1.165)	(1.000)	(1.250)	1.219
3			–	1.221	1.261	(1.168)	(1.083)	(1.083)
4				–	1.321	1.151	(1.083)	(1.500)
5					–	1.191	1.220	(1.165)
6						–	1.050	1.060
7							–	1.089
8								–
RFC								
1	–	2.316	1.936	(2.333)	(2.585)	(2.000)	1.603	2.159
2		–	1.958	2.104	(1.835)	(2.918)	(1.833)	2.180
3			–	1.725	1.481	(3.000)	(1.000)	(2.250)
4				–	1.627	2.195	(1.000)	(1.833)
5					–	1.950	1.147	(1.748)
6						–	1.715	2.270
7							–	1.467
8								–
8								–
Y								
1	–	4.120	3.204	(4.103)	(**4.960**)	(4.500)	4.142	3.502
2		–	3.989	4.016	(4.583)	(4.375)	(**5.250**)	4.287
3			–	3.101	2.758	(4.250)	(4.373)	(3.833)
4				–	2.785	3.785	(4.500)	(3.710)
5					–	3.442	3.695	(2.875)
6						–	4.695	4.056
7							–	4.309
MC								
1	–	3.524	3.138	(3.500)	(3.248)	(4.335)	3.528	3.001
2		–	3.242	3.935	(3.833)	(3.835)	(3.833)	3.105
3			–	3.549	2.763	(3.753)	(3.165)	(2.753)
4				–	3.456	4.188	(4.335)	(3.583)
5					–	3.402	3.152	(2.750)
6						–	3.884	3.358
7							–	3.108
8								–

(Continued)

TABLE 9 | Continued

Parent[a]	1	2	3	4	5	6	7	8
MU								
1	–	3.012	2.885	(3.415)	(2.835)	(2.835)	3.275	3.124
2		–	2.611	2.749	(2.835)	(2.918)	(3.083)	2.850
3			–	2.622	2.422	(2.750)	(3.168)	(2.833)
4				–	2.560	2.820	(3.083)	(2.918)
5					–	2.621	2.812	(2.418)
6						–	3.073	2.921
7							–	3.113
8								–
RFS								
1		1.897	2.137	(2.000)	(2.168)	(2.083)	2.008	2.107
2			2.128	2.017	(1.918)	(2.333)	(1.918)	2.097
3				2.257	2.439	(2.415)	(2.083)	(2.585)
4					2.328	2.322	(2.085)	(2.250)
5						2.505	2.310	(2.250)
6							2.330	2.403
7								2.208
8								
CLR								
1	–	1.738	2.027	(2.000)	(1.750)	(1.585)	1.715	1.766
2		–	2.038	1.882	(1.585)	(1.750)	(1.750)	1.777
3			–	2.171	2.013	(1.833)	(1.918)	(2.000)
4				–	1.857	1.856	(1.835)	(1.835)
5					–	1.698	1.701	(1.753)
6						–	1.699	1.750
7							–	1.753
8								–
BES								
1	–	2.817	3.249	(3.168)	(3.085)	(2.415)	3.186	2.826
2		–	2.904	2.425	(2.165)	(2.585)	(3.165)	2.480
3			–	2.857	2.766	(2.833)	(3.500)	(2.835)
4				–	2.287	2.500	(2.248)	(2.503)
5					–	2.409	2.703	(2.165)
6						–	2.916	2.556
7							–	2.850
8								–
LM								
1		1.234	1.207	(1.250)	(1.333)	(1.418)	1.246	1.206
2			1.201	1.197	(1.583)	(1.248)	(1.083)	1.199
3				1.171	1.315	(1.168)	(1.585)	(1.165)
4					1.312	1.180	(1.500)	(1.182)
5						1.325	1.354	(1.165)
6							1.223	1.182
7								1.212
8								

[a] *1, Paraíso MG H419-1; 2, Catiguá MG2; 3, Oeiras MG 6851; 4, H484-2-18-12; 5, UFV 311-63; 6, Arara; 7, Acauã Novo; 8, Siriema.*

[b] *Vig, vegetative vigor; SC, sprout color; RFC, ripening fruit color; Y, yield; MC, maturation cycle; MU, maturation uniformity; RFS, ripening fruit size; CLR, incidence of coffee leaf rust; BES, incidence of brown eye spot; LM, leaf miner infestation. The highest means for the main traits that were statistically significant are highlighted in bold.*

TABLE 10 | Means of five agronomic traits, evaluated in F_1 hybrids (H1–H12) obtained in a circulating diallel scheme and the respective parents of arabica coffee trees, in Viçosa, state of Minas Gerais, Brazil.

Genotype	Vig[b]		SC		Y		MC		CLR	
H1[a]	6.418	abc[c]	2.333	ab	4.103	abc	3.500	abcd	2.000	ab
H2	7.503	ab	2.585	b	4.960	a	3.248	abcd	1.750	ab
H3	7.750	ab	2.000	ab	4.500	ab	4.335	a	1.585	b
H4	8.085	a	1.835	ab	4.583	ab	3.833	abc	1.585	b
H5	7.250	ab	2.918	a	4.375	abc	3.835	abc	1.750	ab
H6	7.418	ab	1.833	ab	5.250	a	3.833	abc	1.750	ab
H7	6.748	abc	3.000	a	4.250	abc	3.753	abc	1.833	ab
H8	6.500	abc	1.000	b	4.373	abc	3.165	abcd	1.918	ab
H9	6.750	abc	2.250	ab	3.833	abc	2.753	acd	2.000	ab
H10	7.168	ab	1.000	b	4.500	ab	4.335	a	1.835	ab
H11	6.833	abc	1.833	ab	3.710	abc	3.583	abcd	1.835	ab
H12	6.500	abc	1.748	ab	2.875	abc	2.750	bcd	1.753	ab
Mean[c]	7.077		2.028		4.276		3.577		1.800	
Paraíso MGH419-1	4.458	d	2.000	ab	1.558	bc	3.208	abcd	1.708	ab
Catiguá MG2	7.208	ab	2.000	ab	4.438	ab	3.208	abcd	1.793	ab
Oeiras MG 6851	5.000	cd	1.000	b	2.418	abc	2.750	bcd	2.498	a
H484-2-18-12	6.043	bcd	2.000	ab	2.305	abc	4.125	ab	2.000	ab
UFV 311-63	5.833	bcd	1.000	b	1.043	c	2.165	d	1.750	ab
Arara	6.333	abcd	2.000	ab	4.040	abc	3.750	abc	1.835	ab
Acauã Novo	6.625	abc	1.125	b	4.375	abc	3.375	abcd	1.750	ab
Siriema	6.625	abc	1.833	ab	3.375	abc	2.418	cd	1.875	ab
Mean	6.016		1.620		2.944		3.125		1.901	

[a] *H1, Paraíso MG H419-1 × H484-2-18-12; H2, Paraíso MG H419-1 × UFV 311-63; H3, Paraíso MGH419-1 × Arara; H4, Catiguá MG2 × UFV 311-63; H5, Catiguá MG2 × Arara; H6, Catiguá MG2 × Acauã Novo; H7, Oeiras MG 6851 × Arara; H8, Oeiras MG 6851 × Acauã Novo; H9, Oeiras MG 6851 × Siriema; H10, H484-2-18-12 × Acauã Novo; H11, H484-2-18-12 × Siriema; H12, UFV 311-63 × Siriema.*

[b] *Vig, vegetative vigor; SC, sprout color; RFC, ripening fruit color; Y, yield; MC, maturation cycle; CLR, incidence of coffee leaf rust.*

[c] *Means followed by the same letter in the column do not differ statistically by the Tukey test, at 5.0% probability.*

their parents. Hybrids with better MU than both parents were obtaining with the crosses Paraíso MG H419-1 × H484-2-18-12 and Oeiras MG 6851 × Acauã Novo. Higher fruit size was found only with the hybrid from Oeiras MG 6851 × Siriema. In general, the incidence of disease (CLR and BES) and infestation of LM were higher in the hybrids comparing with one of their parents.

Correlation Analysis

Correlation analysis allowed us to verify the relationship between the performance mean of the hybrids analyzed in the diallel model, considering each of the 10 morpho-agronomic traits and the parental SCA, PD, and GD (based on molecular markers).

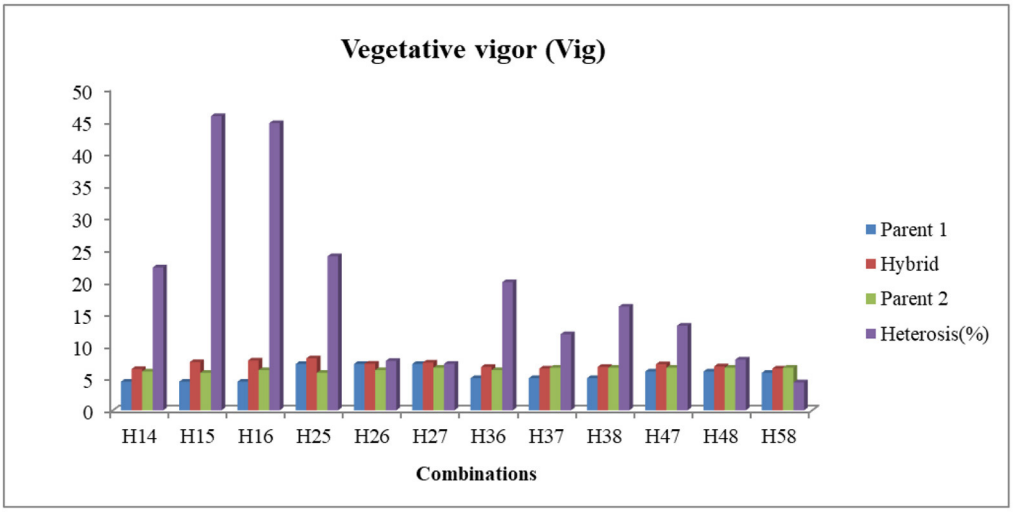
The maximum score obtained from the correlation index was 0.93 (**Figure 4**). In general, PD was not correlated linearly with GD. Phenotypic diversity was also poorly correlated with SCA and the mean performance of the hybrids. The average score of the traits displayed a higher correlation with GD than with PD. Specific combining ability and the means of the hybrids for Vig and Y traits displayed the highest positive correlations with GD. In addition, the CLR trait was highly negatively correlated with Vig trait.

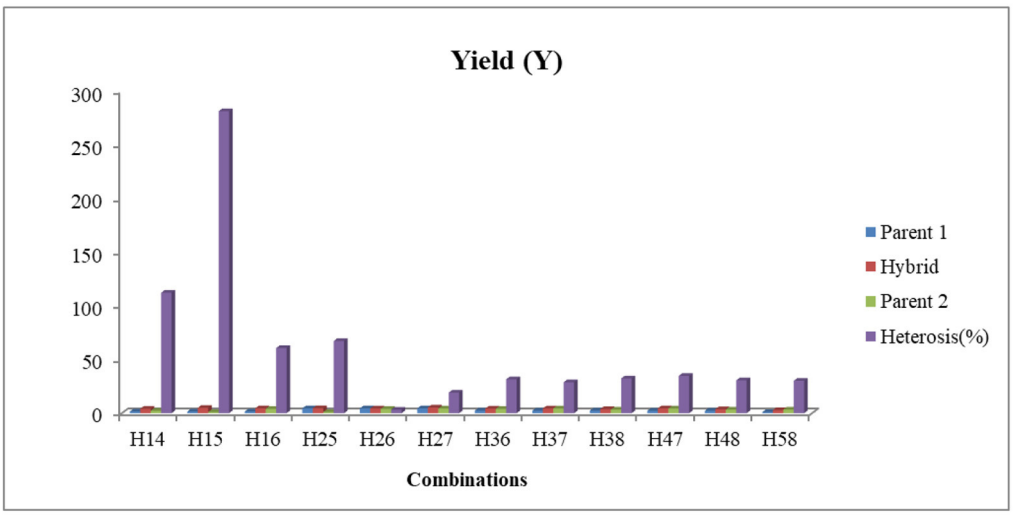
DISCUSSION

Genetic Diversity

The success of crop breeding programs lies in the efficient identification and incorporation of GD, while preserving the important economic traits of an individual plant (Swarup et al., 2020). To achieve this goal, breeders usually use cross-cultivated genotypes to avoid the linkage drag of wild genetic material, but they need to maintain diversity to address the producer and consumer demands. In addition, higher GD in plants allows them to adapt to sudden environmental changes (Raza et al., 2019).

In this study, the DNA of 22 coffee cultivars and accession, with the potential to be used in breeding, showed low diversity (mean: 2.3 bands/primer). The narrow genetic base of *C. arabica* has been reported worldwide, which is explained by the recent origin of the species with a single polyploidization event, autogamous reproduction system, and poor initial global distribution (Missio et al., 2011; Sousa et al., 2017a; da Silva et al., 2019; Jingade et al., 2019; Merot-L'anthoene et al., 2019; Sánchez et al., 2020; Scalabrin et al., 2020). Moreover, its genetic resources have been conserved in the field and, therefore, may be quickly eroded due to local hazards and global climatic change, worsening the genetic variability reduction (Legesse, 2020). For Brazilian cultivars analyzed in this study, the limitation of low





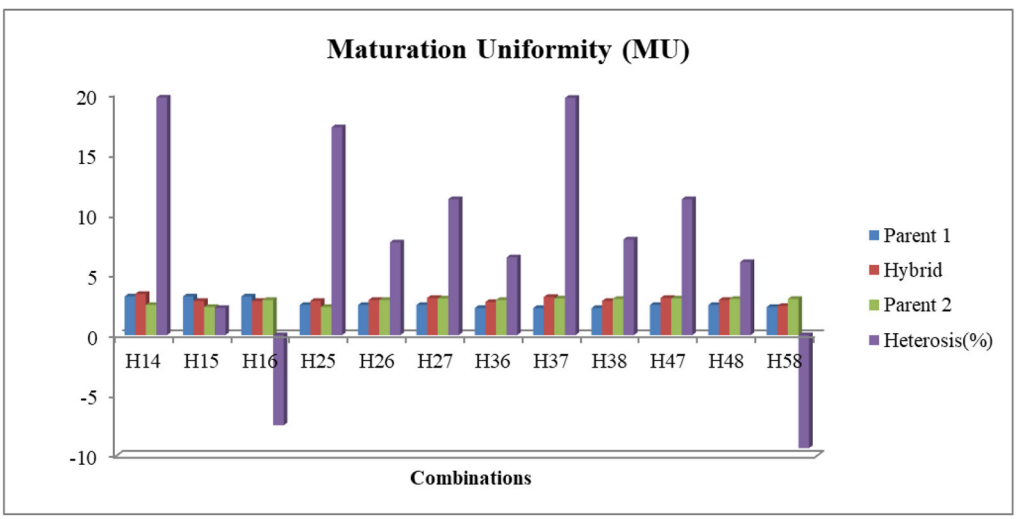
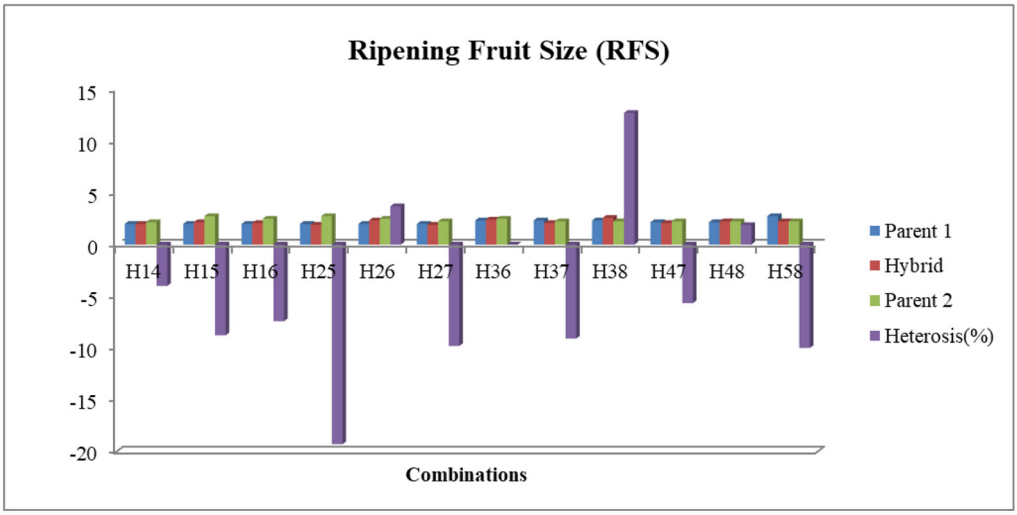
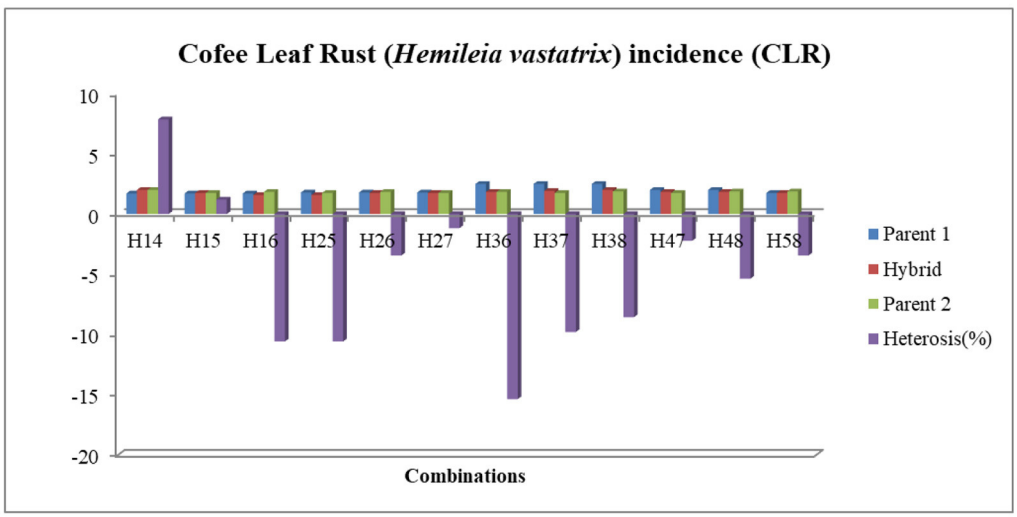


FIGURE 3 | Continued





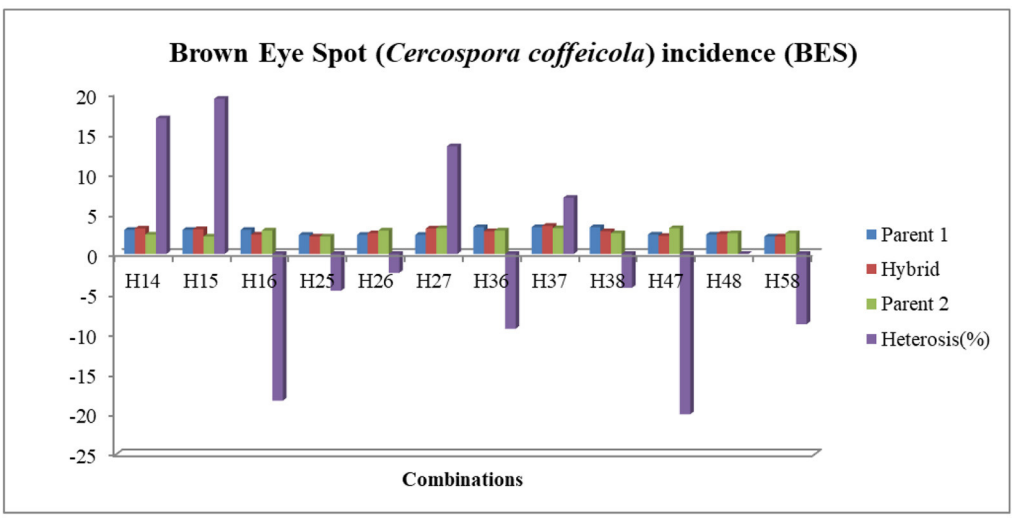


FIGURE 3 | Continued

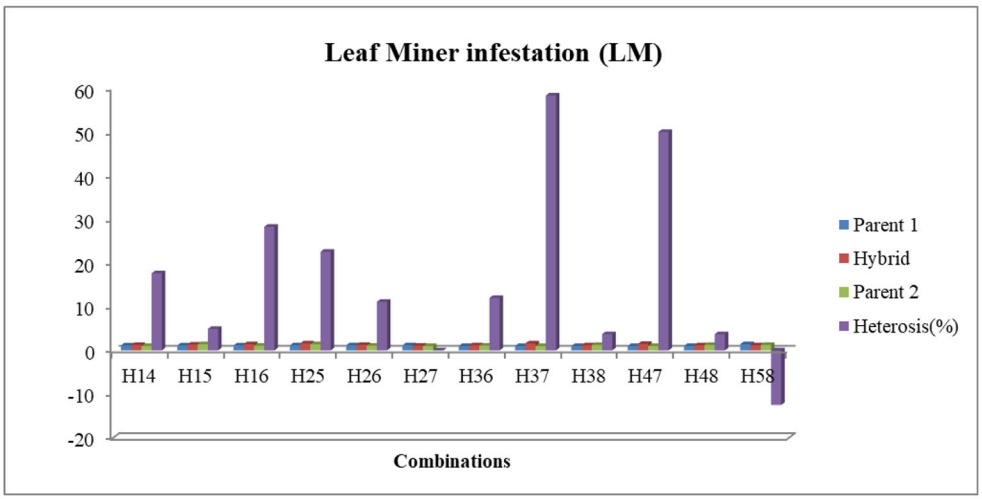


FIGURE 3 | Estimated means of parents and hybrids and values for the heterosis of 12 F_1 hybrids (H14 to H58—**Table 3**) obtained in a circulating diallel scheme, based on the means of agronomic traits.

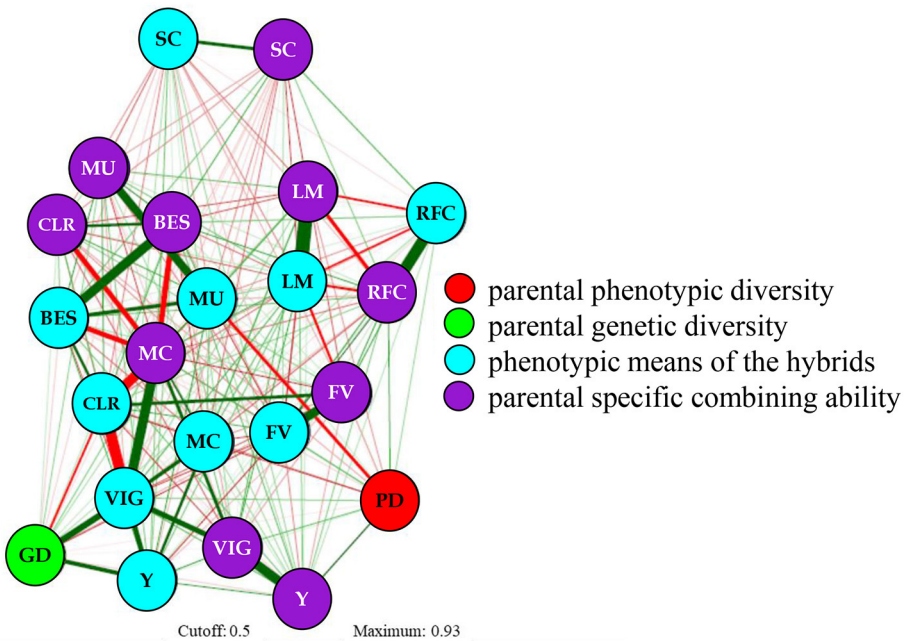


FIGURE 4 | Network correlation showing the relationship among the phenotypic diversity (PD) of the diallel parents, the genetic diversity (GD) of the parents based on SSR markers, the mean performance of the hybrids for each trait, and the specific combining ability. Evaluated traits: vegetative vigor (Vig), sprout color (SC), ripening fruit color (RFC), yield (Y), maturation cycle (MC), maturation uniformity (MU), ripening fruit size (RFS), incidence of coffee leaf rust (CLR), incidence of brown eye spot (BES), and leaf miner infestation (LM). The green lines correspond to the positive correlations and the red lines to the negative correlations. The width of the line is proportional to the intensity of the correlation.

variability has been aggravated by the low number of plants introduced in the country and used in genetic breeding. It has been demonstrated that Brazilian *C. arabica* cultivars originate from a few parents (Setotaw et al., 2013). The genetic base of 121 cultivars released in Brazil between 1939 and 2009 was defined by 13 ancestors, among which seven ancestors contributed 97.55% genetic base. Low genetic variability of the 34 main *C. arabica* cultivars planted in Brazil has been confirmed in another study (Sousa et al., 2017b).

Even with the recognized narrow genetic base of *C. arabica* plants available to be used in the breeding programs, informative data on GD was obtained through molecular markers, genetic distance matrix analysis, and dendrograms in this study. Bourbon Amarelo MG 0009, Topázio MG 1190, Catuaí Amarelo IAC

62, Ibairi IAC 4761, and Catuaí Vermelho IAC 144 were allocated to subgroup I.b (**Figure 1**). This can be explained by the fact that these cultivars/accessions are susceptible to CLR (Legesse, 2020) and do not have the introgression of genes from other coffee species. Genes that confer resistance to CLR and other diseases and pests have been introgressed into *C. arabica* cultivars through interspecific hybrids. The chromosomal section responsible for resistance introgressed in the cultivar and is responsible for increasing the GD (Setotaw et al., 2013, 2020), which explains the separation of cultivars with no interspecific genome. The exception was observed for the cultivar Arara and accession UFV 311-63, which were allocated in subgroup I.b, but they are considered rust-resistant. Arara originated from the spontaneous hybridization between Obatã IAC 1669-20 and Catuaí Amarelo. Obatã IAC 1669-20 itself probably originated from the spontaneous hybridization between Sarchimor and Catuaí (Pereira and Oliveira, 2015). Successive backcrosses with cv. Catuaí may explain the genetic similarity to susceptible coffee trees.

The remaining cultivars/accessions were rust-resistant and distributed in the other groups of the dendrogram, except for the cultivar Arara and accession UFV 311-63, which were allocated in subgroup I.b, the group of rust-susceptible cultivars. Rust-resistant *C. arabica* cultivars are generally derived from the HdT or Icatu interspecific hybrids between *C. arabica* and *C. canephora* (Del Grossi et al., 2013). These germplasms carry genes from the *C. canephora* species, which facilitates *C. canephora* genome introgression in *C. arabica* (Sousa et al., 2017a; Setotaw et al., 2020). These results demonstrate the potential of HdT and Icatu to expand the genetic base of *C. arabica*.

The high dissimilarities in scores between Catiguá MG2 and Acauã cultivars (0.750), and Catiguá MG2 and Topázio MG 1190 cultivars (0.696) can be explained by the parents of these cultivars, which are genetically distant. Catiguá MG2 was originated by crossing Catuaí Amarelo IAC 86 and HdT UFV 440-10, while Acauã originated from the cross between Mundo Novo IAC 388-17 and Sarchimor IAC 1668 (Carvalho, 2008). Topázio MG 1190 originated from the cross between Catuaí Amarelo and Mundo Novo, and therefore does not present *C. canephora* introgression in the genome (Legesse, 2020). Catiguá MG2 showed relatively high genetic distance score (>0.464) with all cultivars/accessions analyzed, except Híbrido de Timor MG 0357 (0.214). This HdT corresponds to the seed obtained from accession HdT UFV-441, which has a high genetic similarity with HdT UFV-440, the progenitor of the cultivar Catiguá MG2 (Silva et al., 2018; Setotaw et al., 2020).

The polymorphism observed in the 76 *C. arabica* plants evaluated in our study, including the polymorphism within cultivar, must be considered for parent selection for the *C. arabica* breeding programs. This information can be used to choose cultivars and individuals within cultivars to be crossed to explore the existing genetic variability and complementarity. For example, if a cross between IAC 125 RN and HdT MG 0357 is the choice due to the complementarity of interest traits, the HdT MG 0357 plant n° 51 should not be selected, as it has been allocated to some IAC 125 RN plants. In contrast, plant n° 51 of HdT MG 0357 can be used, as they were allocated to different groups. These results demonstrate the high efficiency of SSR markers in assisting the selection of the best plants within each cultivar/accession, avoiding the selection of genetically similar plants.

Hybrid Identification by Molecular Markers

Coffea arabica plants were crossed considering their diversity and complementary traits and potential F_1 hybrids were obtained for *C. arabica* breeding. Before advancing to the next breeding generation, the true hybrids were identified. Confirmation of cross success and discrimination between parent genotypes and hybrids is essential for genetic breeding. During breeding, the obtained hybrids are prone to contamination by out-crossing with foreign pollen or physical admixtures (Carvalho, 2008; Krishna et al., 2020). In autogamous species, such as *C. arabica*, self-pollination is common before controlled out-crossing, preventing the transfer of desired traits in progenies. Therefore, cross certification and genetic purity testing of hybrids is a routine and essential approach to an efficient breeding program. Early hybrid identification is a limitation for coffee breeding (Sánchez et al., 2020), and molecular marker analysis can facilitate this process.

In this study, SSR markers were used to certify artificial crossing in a diallel scheme, and the desired crosses were detected in most analyzed hybrids. These results show the informative power of SSR markers in crossbreeding certification. This certification is of great importance in breeding programs, particularly of perennial species, such as *C. arabica*, as it eliminates unwanted genotypes and self-fertilized progenies early, thus saving time, financial resource, and labor.

Although the hybridizations were artificially made by hand, self-fertilized progenies were identified. Hybridization in autogamous plants generally consists of emasculating the flowers and removing the anthers few days before pollination (Georget et al., 2019). In *C. arabica*, the flowers are hermaphrodite and autogamy occurs due to the phenomenon of cleistogamy, which occurs before the flower opens. In this case, emasculation must be performed before the flower opens and before it has been self-pollinated; however, the stigma must already be ripe. Thus, emasculation or crossing was probably performed after self-pollination in the self-fertilized F_1 progenies.

Diallel Analysis

To efficiently select the best crosses for coffee breeding, a diallel analysis was performed in addition to testing the parent diversity, with eight coffee cultivars/accession. The diallel results also revealed the gene action involved in important agronomic characteristics. The variance analysis carried out for 10 morpho-agronomic traits showed additive and non-additive genetic variability, which were statistically significant for both GCA and SCA. For all evaluated traits, the GCA was higher than the SCA, indicating high contribution of the additive gene action in controlling the traits. The additive genes affect the proportion of phenotypic variation transmitted to successive generations and are therefore responsible for the performance of the genotypes in the progeny at when homozygosity is reached (Silva et al., 2013). In autogamous breeding programs, plant selection is practiced in advanced generations of self-fertilization, maximizing genetic progress through the additive effects of genes (Hallauer et al., 2010). Classical *C. arabica* breeding is composed of genealogy and

backcross breeding methods, and the mating system is applied for effective pure-line selection from selfing and elite genotype testing (Fanelli Carvalho et al., 2020). Thus, in autogamous species, such as *C. arabica*, GCA is important for breeders because it depends on additive variance, while SCA depends on the variance due to deviations in dominance.

Low positive or negative GCA estimate indicates that the parental GCA does not differ from the general average. However, when these estimates are high, the parent is superior to the other parents of the diallel. Thus, to develop the hybrid, the best combination should be those with high SCA estimates, whose parents have a high GCA estimate (Kaushik and Dhaliwal, 2018). In this study, Catiguá MG2 and Arara had high GCA estimates for Vig trait (0.494 and 0.299, respectively), indicating that these genotypes are the most recommended for developing a base population for breeding aimed at enhancing Vig. However, the cross between these two parents produced a hybrid with a negative SCA score. Thus, crossing Catiguá MG2 and UFV 311-63 should be prioritized for Vig improvement, as both parents presented positive GCA estimates and high SCA effect. The diallel analysis also showed that the combination of Paraíso MG H 419-1 and Arara has potential for Vig breeding as it presented a high SCA effect score (1.278) and involves one of the parents with high GCA estimate (Arara). Vig is an important trait for coffee, as it is positively correlated with Y (Severino et al., 2008; Pedro et al., 2011) and genotype adaptation, reflecting less depleted plants (Nadaleti et al., 2018).

Like other crops, one of the main objectives of coffee breeding programs is to increase productivity (De Paiva Barbosa et al., 2019a). Therefore, based on diallel data, Acauã Novo and Catiguá MG2 had the highest GCA scores for Y (0.603 and 0.581, respectively). The hybrid from the cross between these two parents showed an estimated SCA score of 0.324. Thus, as the estimated SCA score for this hybrid was high and positive and involved the two parents with the highest GCA scores, the cross between Catiguá MG2 and Acauã Novo is recommended for this trait. Another potential cross would be between the Catiguá MG2 and UFV 311-63, since the hybrid resulting from this cross had the highest estimated SCA score (0.909) and involved one of the parents with high estimated GCA score.

UFV 311-63 and Oeiras MG 6851 displayed the best GCA scores for fruit MU (−0.241 and −0.179, respectively) and no significant difference in the SCA scores. The lowest negative scores are desirable for MU, since the lowest scores indicate the highest fruit ripening uniformity. MU is related to coffee beverage quality, and in the recent years, there has been an increasing demand for the best quality coffees (De Paiva Barbosa et al., 2019a); moreover, MU contributes to labor reduction during harvest.

In addition to yield and quality, coffee breeding focuses on biotic stress resistance. In this study, we evaluated the parental resistance to CLR, BES, and leaf miner. Phenotypic evaluation was based on the incidence and infestation of these biotrophic agents. Thus, the plants that received the lowest score were the most resistant, implying that low GCA and SCA scores are desired. Arara and UFV 311-63 displayed the best GCA scores for CLR (−0.072 and −0.070, respectively), and no significant difference in the SCA scores. Thus, crosses involving UFV 311-63 and Arara have the greatest potential for resistance to CLR. UFV 311-63 and H 484-2-18-12 displayed the best GCA scores for BES (−0.268 and −0.178, respectively), and no significant difference in the SCA scores was obtained. Crosses involving at least one of these parents are recommended. Leaf miner infestation was similar in all parents studied.

The hybrids with the highest scores of phenotypic mean for Vig and Y traits were obtained from the recommended crosses based on GCA and SCA evaluation (Catiguá MG2 × UFV311-63 for Vig and Catiguá MG2 × Acauã Novo for Y). Moreover, the mean yield of these hybrids was higher than that of their parents. This result shows that the combining ability evaluation-based hybrid prediction is an effective technique and can be particularly useful for breeding programs, since cross selection in the diallel scheme was based on genetic parameters.

In general, crosses involving Catiguá MG2, UFV 311-63, and Arara were the most promising. Hybrids originating from Catiguá MG2 were recommended for Vig and Y traits, and this cultivar also showed the highest genetic distance from almost all other *C. arabica* progenies analyzed according to the GD study (**Figures 1**, **2**). These results show a potential relationship between GD, phenotypic mean, and combining ability. In addition, Catiguá MG2 has been used as a source to enhance cup quality and obtain specialty coffee (Alex et al., 2016; De Paiva Barbosa et al., 2019b). This cultivar has also been highlighted for its high resistance to CLR (Del Grossi et al., 2013) and moderate resistance to bacteriosis caused by *Pseudomonas syringae* (Fernandes et al., 2020).

As the crosses were made between fairly inbred parents, the heterosis was also evaluated for the different traits. Hybrid vigor or heterosis represents the average superiority of a crossbred individual in relation to the average performance of their parents. Heterosis also depends on genetic differences between the parents being crossed. In our work, hybrids that surpassed the average of their parents in some traits were identified. Other studies showed the advantages of exploiting heterosis in *C. arabica* hybrids, with significant yield gains, when compared to the average of the parents and the best parent (Bertrand et al., 2005, 2011). However, no hybrid in our work showed superiority for all evaluated agronomic characteristics. A higher level of heterosis was found for Vig and Y and, in general, the cultivar Paraíso MG H419-1 was used as one of the hybrids parentals.

Correlation Analysis

In breeding programs, genetically contrasting cultivars/accessions containing relevant agronomic traits are mostly crossed. Thus, through gene recombination, it is possible to obtain genetic gains that enable the success of breeding programs. Nevertheless, the ideal situation is the involvement of genetically diverse parents with good proven performance in crosses, whenever possible (Ramalho et al., 2013). To assist this strategy, it is important to evaluate cultivars and elite accessions and test the relationship among their GD (obtained here by molecular markers), PD, phenotypic mean of their hybrids, and combining ability. Thus, if these parameters are somehow correlated, a complex parameter should be estimated using a simple and low-cost analysis. For example, for Vig and yield (Y), the cross between Catiguá MG2 and UFV 311-63

has recommended based on the scores of general and specific combining abilities obtained through diallel analysis. Genetic distance matrix analysis indicated that these two genotypes had a high genetic dissimilarity score (distance = 0.589).

The joint analysis of molecular and phenotypic data was especially important in our work. Diallel analysis were performed based on phenotypic data from a single year, the first year with significant yield (3.4 years after planting). Since some evaluated traits are quantitative, data from additional years would be required to confirm the performance of hybrids and parentals. However, the high correlation between GD and phenotypic data allowed the early selection of genetic materials, which is essential for coffee, a perennial crop with a long reproductive cycle.

Network correlation indicated that PD was not linearly correlated with GD in the eight *C. arabica* plants evaluated using circulating diallel (**Figure 4**). Phenotypic diversity was also poorly correlated with specific combining abilities and mean performance of hybrids in all evaluated traits. However, hybrid performance and parent-specific combing ability were closely correlated with GD. These results corroborate those obtained for corn (Makumbi et al., 2011) and sunflower (Reif et al., 2013). The correlation between molecular marker-based GD and combining ability was evaluated in genetically related and unrelated groups of sunflowers *(Helianthus annus L.)* (Reif et al., 2013). A strong correlation was observed for related genotypes, but not for unrelated genotypes. For tropical test lines of corn *(Zea mays L.)*, a high correlation was also observed when related genetic materials within the same heterotic group were crossed.

Previous studies have demonstrated a high correlation between the combination ability estimated using diallel crosses and genetic distance estimated using molecular markers, when genetically related genotypes are crossed. *Coffea arabica* plants are genetically related due to their narrow genetic base and, therefore, reduced genetic variability (Sousa et al., 2017a), which can maximize the correlation between these parameters. The correlation among molecular GD, hybrid performance, and SCA can help in cultivar selection for the genetic improvement of *C. arabica*. The use of molecular GD allows the early selection of *C. arabica* plants in the breeding program, which is particularly important for perennial and long-cycle species (Sousa et al., 2019).

Vig and Y traits were the most positively correlated with GD. In addition, CLR incidence trait was highly negatively correlated with Vig trait. These traits have been the focus of most coffee breeding programs. The 12 SSR markers used to assess GD were pre-selected in other studies (Pestana et al., 2015; Sousa et al., 2017b) because they are highly polymorphic, assuming high importance in this species due to their narrow genetic basis. Thus, it can be inferred that GD evaluation-based parent selection for the genetic improvement of *C. arabica* is a useful technique, since the molecular distance was highly correlated with the phenotypic means of the hybrids and parent combining ability.

CONCLUSIONS

Molecular marker-based GD analysis allows a detailed assessment of the genetic distance between and within coffee cultivars/accessions. Using molecular markers is an efficient approach to assist parent selection and true hybrid identification to develop a segregating *C. arabica* population for breeding and therefore efficiently increase GD in *C. arabica* cultivars. Circulating diallel cross is an effective technique for the genetic improvement of *C. arabica*, and selecting crosses based on general and specific combining abilities can be very useful for obtaining promising breeding population.

Coffea arabica breeders can increase GD through strategic molecular marker integration, crossbreeding certification, and diallel approach, while preserving the economically important traits of individual crops. Using this strategy, elite genetic resource can be included in breeding programs and new cultivars may be developed in response to rapid shifts in global coffee cultivation conditions and resources due to climate change and new demand from coffee producers and consumers.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

EC and AO: conceptualization. AM, TS, and CC: methodology. CC: software. EC, AO, LZ, and AP: validation. AM and EC: formal analysis and investigation. AO and AP: resources. AM, EC, TS, and VS: writing—original draft preparation and review and editing, EC: supervision. EC, AO, LZ, and AP: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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Agro-Ecological Management of Coffee Pests in Brazil

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Coffee plants host several herbivorous species, but only few are considered pests. Brazil is the largest coffee producer of the world, and the two key coffee pests of the crop in the country are the coffee leaf miner *Leucoptera coffeella* and the coffee berry borer *Hypothenemus hampei*. However, in some regions or on specific conditions, species of mites and scales can also cause damage to coffee plants. Conventional management of coffee pests relies on chemical pesticides, and it is the most commonly used strategy in Brazil, but environmental problems, pest resistance, and toxicity-related issues have led coffee growers to search for alternatives for pest control. Agro-ecological strategies suitable to coffee cultivation can be adopted by farmers, based on plant diversification, in order to provide resources for natural enemies, such as nectar, pollen, shelter, microclimate conditions, and oviposition sites, thereby promoting conservation biological control. Here I revise these strategies and report the results from research in Brazil. I include results on agroforestry, use of cover crops, and non-crop plant management. These are complemented by curative measures based on the use of organic farming-approved pesticides that can be employed when the agro-ecological practices are not yet consolidated. I also present the cultural control method used by several coffee producers in Brazil to decrease coffee berry borer damage.

Keywords: conservation biological control, coffee berry borer, coffee leaf miner, biopesticides, cultural control

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INTRODUCTION

Coffee (*Coffee arabica* L. and *Coffee canephora* L) (Rubiaceae) can host at least 850 insect species, but only few are considered major pests (Le Pelley, 1968). Among those, the coffee leaf miner *Leucoptera coffeella* (Guérin-Mèneville) (Lepidoptera: Lyonetiidae) and the coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae) stand out as key pests in Brazil, the largest coffee producer of the world (Le Pelley, 1968; Reis et al., 2002; Vega et al., 2009). Pest attack on coffee plants causes losses of hundreds of millions of dollars every year (Oliveira et al., 2013; Milligan et al., 2016; Avelino et al., 2018; Cure et al., 2020). The coffee leaf miner (CLM) is disseminated throughout the American continent (Pantoja-Gomez et al., 2019). Its development and reproduction are favored by hot and dry climate conditions, which are found in most regions where coffee is cultivated in Brazil (Silva et al., 2014; Giraldo-Jaramillo et al., 2019; Leite et al., 2020). Females of CLM lay their eggs on the adaxial leaf surface of coffee plants, and the larvae feed on the cells of palisade parenchyma, causing leaves to dry and to prematurely fall (Reis and Souza, 1996; Reis et al., 2002). At high population levels, CLM may cause defoliation up to 70%, which decreases photosynthesis and results in up to 50% decrease in coffee yield (Reis and Souza, 1996).

The coffee berry borer (CBB) is a cosmopolitan pest currently present in all coffee producer countries except in Australia and Nepal (Johnson et al., 2020; Sun et al., 2020). It is considered the most damaging pest of coffee worldwide (Damon, 2000; Vega et al., 2009; Cure et al., 2020). Females of CBB bore into the berries and oviposit inside the coffee berry endosperm. The hatched larvae feed on the seeds, resulting in losses of quality and quantity of the marketable coffee (Damon, 2000; Jaramillo et al., 2006; Vega et al., 2009). It is the only species that can feed and complete its cycle on coffee seeds due to the bacterial symbiotes in its gut that degrade caffeine (Ceja-Navarro et al., 2015; Vega et al., 2021). In addition to CBB and CLM, mites and scales can also cause damage to coffee plants, leading to yield reduction (Reis et al., 2002). The red mite, *Oligonychus ilicis* (McGregor) (Acari: Tetranychidae), is found on the upper coffee leaf surface where it punctures the epidermis and mesophyll cells of the leaf and absorbs the cell contents, resulting in bronze-colored leaves, which can reduce the photosynthesis rate by up to 50% (Franco et al., 2008). The infestation usually occurs in patches, but it may spread over the entire crop, mainly in the dry periods. Scales from Coccidae [*Coccus viridis* (Green)] and Pseudococcidae [*Planococcus citri* (Risso)] families cause damage to coffee plants due to their feeding on the plant sap and to the injection of toxins into the vascular system (Santa-Cecília et al., 2002; Fernandes et al., 2009; Rosado et al., 2013).

The use of pesticides is the most common control measure for coffee pests in Brazil. For instance, at higher infestations of CLM, the number of sprayings can reach up to 20 per year (Leite et al., 2020). The use of pyrethroids for CLM control has been related to increased phytophagous mite outbreaks, mainly due to their deleterious effects on predatory mites (Reis et al., 2007) and to pest-induced hormesis (a stimulating and beneficial effect to living organisms of a harmful substance) under low doses (Cordeiro et al., 2013). Despite the overuse of synthetic pesticides, CBB continues to cause major economic losses (Oliveira et al., 2013; Infante et al., 2014; Johnson et al., 2020). There are many concerns associated with the reliance on pesticide applications, such as harmful effects to human health and to the environment, pest resistance, outbreaks of secondary pest, and loss of beneficial insects (Fragoso et al., 2003; Reis et al., 2015; Hutter et al., 2018; Leite et al., 2020, 2021), which signal the need for developing alternative strategies and discovering new sustainable pest controls. Considering the biology and feeding habits of coffee pests, integrative measures are key to manage them (Damon, 2000; Vega et al., 2009; Infante, 2018; Johnson et al., 2020).

Here I revise the main agro-ecological strategies, focusing on conservation biological control, that could be used for coffee pest management in Brazil. I also report curative measures based on the use of organic farming-approved biopesticides that can be employed when agro-ecological measures are not yet consolidated. Finally, I present and discuss about cultural practices for managing coffee berry borer population. The agro-ecological coffee pest strategies proposed here are based on scientifically reported results. The strategies aim not only coffee productivity but also to recover from the environmental and social harms caused by conventional agriculture and to reduce the dependency on external inputs such as pesticides (Cardoso et al., 2001; Sales et al., 2013). Most of the presented strategies can be adopted and adapted for a range of coffee size farms, but they are especially suitable for small-scale coffee farmers that, in Brazil, are responsible for 80% of coffee production.

CONSERVATION BIOLOGICAL CONTROL

Coffee crops naturally harbor a great diversity of natural enemy species, such as predatory and parasitoid wasps, green lacewings, ants, ladybugs, predatory mites, and entomopathogens (Reis et al., 2002; Fernandes et al., 2008; Amaral et al., 2010; Rodrigues-Silva et al., 2017; Moreira et al., 2019; Botti et al., 2021; Rosado et al., 2021). However, their abundance in coffee monocultures is not always enough to keep pest populations below economic injury levels; for instance, predators and parasitoids, although carnivores, need to feed on plant-derived food, such as nectar and pollen, to supplement or complement their diet or during a non-carnivorous life stage (Olson et al., 2005; Venzon et al., 2006, 2019). In coffee monocultures, these resources are not available throughout the year, as coffee blooms for a limited period and the flowers last only a few days, when they are already visited by pollinators (Peters and Carroll, 2012). Besides alternative food, predators and parasitoids need microclimate conditions, shelter, and place to oviposit and build their nests (e.g., predatory wasps), which are not available in coffee monocultures. These resource provisions can be done by keeping forest fragments near to crop area (Aristizábal and Metzger, 2019; Medeiros et al., 2019), by increasing the plant diversity on it, and on the surroundings by adding trees (i.e., agroforestry), intercropping cover crops, and managing non-crop plants.

More diversified agroforestry systems are usually related to positive effects on coffee pest control (Philpott and Armbrecht, 2006; Philpott et al., 2008; Teodoro et al., 2009; Perfecto et al., 2014; Pumariño et al., 2015). Moreover, selecting tree species for biological control purposes can be optimized based on their interaction with pests and natural enemies (Heil, 2015; Peters et al., 2016). A remarkable example is the coffee agroforestry system in the Atlantic Rainforest Biome (a South American forest that extends along the Atlantic coast of Brazil) where small stakeholders associated coffee to several trees and, among them, nitrogen-fixing species that bear extrafloral nectaries (EFN) (Souza et al., 2010; Rezende et al., 2014) (**Figure 1**). EFN are nectar-secreting glands located outside the flowers, and their presence is common in tropical plants (Koptur, 2005). Nectar from EFN is available along the year, and it is more accessible to natural enemies of pests that usually have short mouthparts. In return to the food provided by EFN, the natural enemies protect the plants against herbivory. Rezende et al. (2014) showed an associational resistance provided by EFN-possessing Inga trees (*Inga* spp.) to coffee plants in agroforestry systems. The EFN of inga attract predators and parasitoids of CLM (**Figures 2A,B**). The parasitism of CLM increased significantly with the abundance of nectary visitors, and the proportion of mined leaves decreased significantly with this abundance. Later, in a replicated field experiment, Rezende et al. (2021)

FIGURE 1 | Agroforestry coffee system in the Atlantic Rainforest Biome, Araponga, state of Minas Gerais, Brazil (photo from Maíra Queiroz Rezende).

confirmed that the damage caused by CLM and CBB was lower in coffee consorted with inga trees than in plots with coffee only. Moreover, the authors show that coffee plants consorted with inga trees produced heavier fruits than unconsorted coffee plants. Furthermore, inga trees mediated CBB predation by hosting a predatory thrips of the genus *Trybomia* (Thysanoptera: Phlaeothripidae) that feeds on CBB eggs, larvae, and pupae (Rezende et al., 2014; Pantoja, 2018). The predator benefits from EFN feeding (**Figure 3**) as its survival increases but still depends on a protein food source to complete its development (Rezende, 2014; Coffler, 2020).

The diversified landscape, microclimatic stability, and reduced soil disturbance in agroforestry coffee systems in the Atlantic Rainforest Biome had a positive effect on the activity and abundance of insect-pathogenic fungi when compared with the full-sun conventional coffee production system (Moreira et al., 2019). Soil from coffee plots diversified with *Inga edulis* Mart. (Fabaceae), *Senna macranthera* (Collad.) Irwin et Barn. (Fabaceae), *Varronia curassavica* Jacq. (Cordiaceae), and non-crop plants at Cerrado (a Savanna-like vegetation and one of the Brazilian biomes) of Minas Gerais harbor a more abundant and diverse community of entomopathogenic fungi species than plots with conventional monoculture (Franzin, M.L, personal communication) (**Figure 4**).

Intercropping coffee with cover crops is another viable strategy to increase the availability of plant-provided food, refuges, and favorable microclimate for predators and parasitoids, enhancing their survival and performance and thereby resulting in increased effectiveness for pest control (Venzon et al., 2006; Amaral et al., 2010; Rosado et al., 2021). Cover crops improve the chemical, physical, and biological characteristics of the soil and contribute to the reduction of the diseases and weeds in coffee crops (Colozzi Filho and Cardoso, 2000; Paulo et al., 2001; Mendonça et al., 2017). Regarding the biological control of coffee pests, Venzon et al. (2006) evaluated the suitability of leguminous cover crop pollens to the green lacewing *Chrysoperla externa* (Hagen) (Neuroptera: Chrysopidae), a common predator species found in coffee agro-ecosystems (Ribeiro et al., 2014; Rodrigues-Silva et al., 2017). Both the adults and larvae of *C. externa* can feed on plant material, while the larvae feed on a variety of soft-bodied arthropods, including the CLM, CBB, mites, and scales (Ecole et al., 2002; Venzon et al., 2009; Rodrigues-Silva et al., 2017; Carvalho et al., 2019; Botti et al., 2021). The presence of alternative plant food sources for lacewings is especially important in times of prey scarcity, allowing their presence in crops even when prey is temporarily unavailable. The pollens of pigeon pea (*Cajanus cajan* (L) Millsp., Fabaceae) and sunn hemp (*Crotalaria juncea* L., Fabaceae) were equally suitable for *C. externa*, especially when they were complemented with a carbohydrate source (Venzon et al., 2006). Combining a plant providing pollen (sunn hemp) and a plant providing nectar (buckwheat, *Fagopyrum esculentum* Moench, Polygonaceae) increased the chances of predator survival (Rosado, 2007) (**Figures 5A,B**). Buckwheat also affords pollen, but it is known by the high nectar productivity of its flowers.

Intercropping buckwheat and sunn hemp with coffee increased the activities of predation and of parasitism, respectively, promoted by wasps on CLM (Rosado et al., 2021) (**Figures 6A,B**). The predation rate of Vespidae on CLM larvae was higher in intercropped plots compared to monoculture, where no other plant food resource was available. Adults of Vespidae forage for nectar and pollen to satisfy their nutritional demands and to feed their larvae (Klein et al., 2002). Both foods

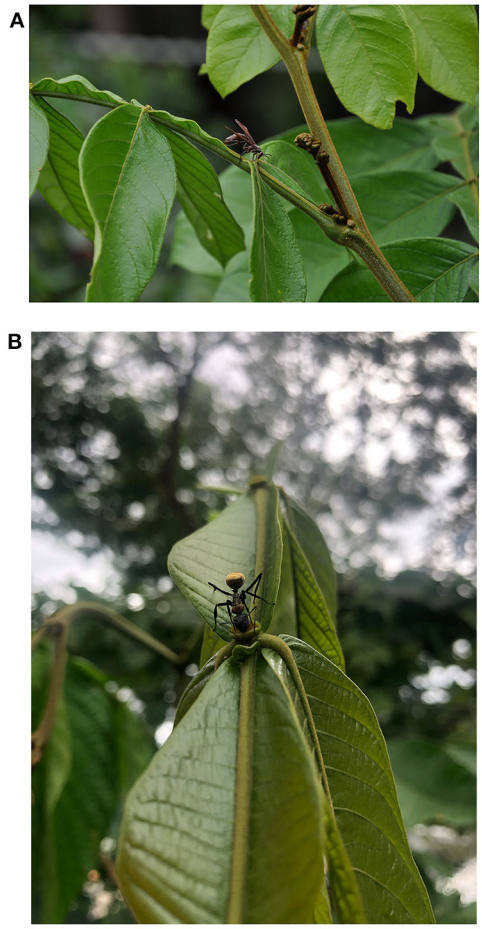

FIGURE 2 | Predators feeding on extrafloral nectar of inga trees: **(A)** Vespidae (photo from Maíra Q. Rezende). **(B)** Formicidae (photo from Madelaine Venzon).

FIGURE 3 | *Trybomia* sp. feeding on extrafloral nectar of inga trees (photo from Madelaine Venzon).

can be found in the large sunn hemp papilionaceous flowers that can be easily accessed by adults of Vespidae (Amaral et al., 2010; Meagher Jr et al., 2019). Buckwheat intercropped with coffee increased the parasitism by Eulophidae and Braconidae in CLM (Rosado et al., 2021) (**Figure 7**). Feeding on buckwheat nectar enhances the survival and reproduction of some parasitoid species of these families (Rosado, 2007; Nafziger and Fadamiro, 2011) (**Figure 8**). Intercropping sunn hemp with coffee increased the population of predatory mites of the Phytoseiidae family and lowered the herbivorous Tetranychidae population compared to coffee monoculture (Rosado et al., 2021). The pollen of sunn hemp is nutritionally suitable to Phytoseiidae (Rodríguez-Cruz et al., 2013).

Non-crop plants (i.e., spontaneous plants) can also be managed for conservation biological control purposes in agro-ecosystems, as they provide resources and conditions that allow natural enemy survival, growth, and reproduction, even when their prey are scarce or absent (Venzon et al., 2019). One of the main advantages of using non-crop plants to habitat manipulation is that they grow rapidly and spontaneously, and generally farmers know them well. However, the effectiveness of this strategy depends on finding the functional role of each plant to specific biological control agents. Chrysopidae larvae can benefit by feeding on flower resources of non-crop plants during periods of prey scarcity. The larvae of *C. externa* had higher survival when tropical ageratum (*Ageratum conyzoides* L., Asteraceae) was offered (**Figure 9**), and the same happens to the larvae of *Ceraeochrysa cubana* (Hagen) (Neuroptera: Chrysopidae) when *A. conyzoides* or beggar tick (*Bidens pilosa* L., Asteraceae) was provided (Salgado, 2014). Coccinellidae was more abundant when aphids were present on non-crop plants, but they were also observed foraging on flowers, EFN, and using the plants as refuge (Amaral et al., 2013) (**Figure 10**). Feeding on flowers of *B. pilosa* increased Coccinellidae survival in the absence of prey (Fonseca et al., 2017). Spiders use non-crop plants as substrate to build webs, mainly on taller and ramified plants (Amaral et al., 2016) (**Figure 11**). Keeping and managing non-crop plants contribute to the maintenance of ants that nest in the ground and are important predators of CBB and CLM (Lomelí-Flores et al., 2009; Larsen and Philpott, 2010; Piato et al., 2021).

Besides the top-down effect on coffee pests mediated by vegetational diversity, as shown above, increasing plant diversity

FIGURE 4 | Coffee plot diversified with *Inga edulis*, *Senna macranthera*, *Varronia curassavica*, and non-crop plants at Cerrado, Patrocínio, state of Minas Gerais, Brazil (photo from Mayara Loss Franzin).

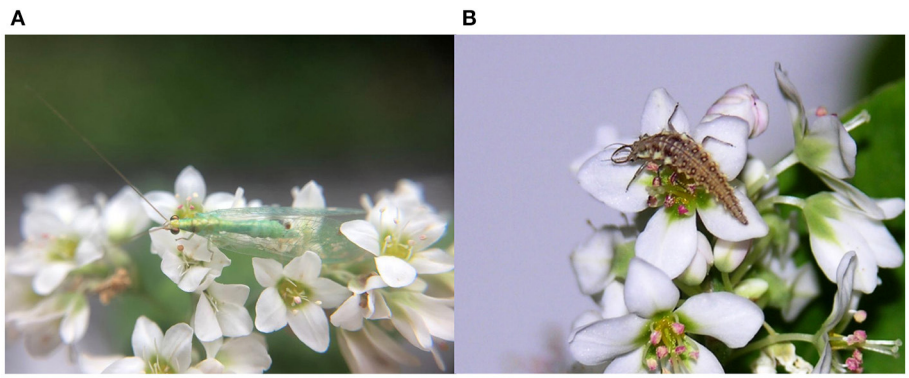

FIGURE 5 | *Chrysoperla externa* feeding on buckwheat nectar and pollen: **(A)** adult and **(B)** larvae (photos from Madelaine Venzon).

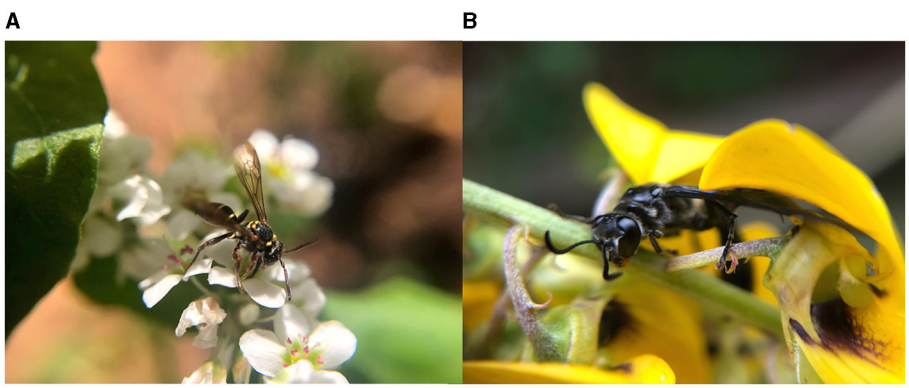

FIGURE 6 | Vespidae on flowers of **(A)** buckwheat and **(B)** sunn hemp (photos from Madelaine Venzon).

FIGURE 7 | Buckwheat intercropped with coffee in the Atlantic Rainforest Biome, São Miguel do Anta, state of Minas Gerais, Brazil (photo from Maria da Consolação Rosado).

FIGURE 8 | Coffee leaf miner parasitoid feeding on buckwheat nectar (photo from Jéssica Mayara Coffer Botti).

FIGURE 9 | *Chrysoperla externa* larva on flower of *Ageratum conyzoides* (photo from Madelaine Venzon).

can have effects on pest populations by modifying the local abiotic parameters that affect pest dynamics (Teodoro et al., 2008; Lomelí-Flores et al., 2010; Avelino et al., 2012; Rice, 2018). Temperature affects CBB infestation (Jaramillo et al., 2009), and trees can effectively reduce the temperature in coffee fields (Mariño et al., 2016; Gomes et al., 2020). Jaramillo et al. (2009) modeled CBB reproduction with temperature and concluded that a 1°C rise in temperature results in 8.5% of average increase on pest population. However, the effect of shade on CBB infestation is not yet clear, as variable results are reported and may partly reflect the varying conditions in the country and areas where the studies have been done (Mariño et al., 2016). In Brazil, there are some preliminary reports about CBB infestation on agroforestry coffee systems, and the effects on CBB range from negative, neutral, and positive (Campanha et al., 2004; Lopes et al., 2012; Figueiredo et al., 2016). Long-term studies in different biomes where coffee is cultivated in Brazil are thus needed. For CLM, intercropping rubber trees with coffee lowered the pest infestation due to microclimate conditions unfavorable to CLM (Androcioli et al., 2018). The authors pointed out that the shade of coffee leaves may result in changes in the leaf structure that may impair CLM survival rate. The use of border crops which act as physical barriers to CBB and CLM flight between coffee areas could also represent a strategy to reduce pest movement (DeClerck et al., 2015).

Several criteria are used when selecting plants for coffee crop diversification. The trees used for diversification of coffee agroforesty systems in Brazil are chosen by family farmers based on compatibility with coffee, biomass production, nitrogen fixation, labor intensity, and diversification of the production (Cardoso et al., 2001; Souza et al., 2010), but as shown above, research has been carried out to select more plant species to be used in coffee agro-ecosystems, with the additional aim of reducing coffee pest populations by increasing the natural enemy populations. One important trait when selecting plants

FIGURE 10 | Coccinellidae larvae on flower of non-crop plant (photo from Madelaine Venzon).

FIGURE 11 | Spider on non-crop plants (photo from Madelaine Venzon).

that provide food to predators and parasitoids is that they should not benefit the herbivores that attack the coffee plants. This is important considering that CLM can feed on nectar. Thus, an assessment of accessibility and suitability of floral nectar is necessary. For buckwheat, laboratory experiments confirmed that its nectar does not benefit the CLM (Rosado, 2007). Furthermore, plants should be employed according to the production system and biome where coffee is cultivated. It is also important to point out that plant diversification in coffee crops enhances pollinator populations, leading to increasing yield (Saturni et al., 2016; Hipólito et al., 2018). Understanding the ecosystem services provided by individual plant species will help in unraveling the mechanisms which enhance pest control in diversified systems and can also help in the design of pest-suppressive coffee systems (Staver et al., 2001; Rezende et al., 2021). The final aim is to diversify coffee agro-ecosystems while ensuring food security, healthy environment, and the economy support of coffee growing families.

CURATIVE PEST CONTROL MEASURES

Curative measures are applied when the preventive tactics fail to restrain the pest population growth (Zehnder et al., 2007). These measures include the use of biopesticides and other non-synthetic products approved by national organic standard organizations.

Biopesticides

Releases of the parasitoid wasp species *Cephalonomia stephanoderis* Betrem (Hymenoptera: Bethylidae), *Prorops nasuta* Wat., and *Phymastichus coffea* LaSalle (Hymenoptera: Eulophidae) showed a variable action on CBB populations, lack of establishment in some of the new world coffee areas and challenges in their mass rearing. Comprehensive reviews about their origin, introduction, and constrains related to their use can be found on revisions done by Vega et al. (2009), Aristizábal et al. (2016), and Johnson et al. (2020). A successful example is from Colombia, where Aristizábal et al. (2012) show that the release of CBB parasitoids in areas without pesticide applications reduced the CBB populations. Possibly, a combination of such releases with conservation biological control strategies would provide a long-lasting CBB population control, but it has to be tested. In Brazil, despite past efforts (Benassi, 1995, 2007), currently there are no reports about rearing and releasing of parasitoids for CBB control.

Predators are the least-studied natural enemies of coffee pests for augmentative control. Notwithstanding the important role of ants, coffee farm workers typically have a negative view of ants due to their aggressiveness during harvesting (Philpott

FIGURE 12 | First-instar larvae of *Chrysoperla externa* feeding on coffee berry borer egg (photo from Jéssica Mayara Coffer Botti).

FIGURE 13 | Second-instar larvae of *Ceraeochrysa cubana* feeding on coffee leaf miner egg (photo from Elem Fialho Martins).

and Armbrecht, 2006; Offenberg, 2015). Other CBB predators reported are species from Thysanoptera (Phlaeothripidae) (Jaramillo et al., 2010; Rezende et al., 2014), Hemiptera (Anthocoridae) (Bustillo et al., 2002), and Coleoptera (Silvanidae, Laemophloeidae, Cucujidae) (Vega et al., 1999; Bustillo et al., 2002; Follett et al., 2016; Sim et al., 2016). Except for bark beetles (Follett et al., 2016), there are no reports about the rearing and releasing of predators for CBB control, but they are important for conservation biological control purposes. Recently, a Chrysopidae species (*C. externa*) was reported to prey on CBB in Brazil. First-instar larvae were able to access CBB galleries, remove pest immature stages, and prey on them (**Figure 12**). Predation by the third instar larvae on CBB adults was also observed (Botti et al., 2021). Additionally, this species and *C. cubana* prey on CLM immature stages (**Figure 13**), on mites, and on scales (Ecole et al., 2002; Venzon et al., 2009; Martins et al., 2021). A reference specification (Togni et al., 2019) needed for the registration process of *C. externa* as a biopesticide was recently published by the Ministry of Agriculture Livestock and Food Supply and will represent a useful tool for coffee pest management (MAPA-Ministério da Agricultura, 2021).

Among biopesticides, the entomopathogenic fungi *B. bassiana* is one of the widespread biopesticides for CBB in Brazil. There are several formulated products that are commercially available. It is now currently used in medium and large coffee farms, and it is beginning to be used in small-scale farms. The efficiency of *B. bassiana*-formulated products is extremely variable and dependent on environmental conditions and on the strain of the pathogen, among other factors (Aristizábal et al., 2016; Johnson et al., 2020). According to Mascarin and Jaronski (2016), *Beauveria* sprayable formulations can be applied for CBB control as conidia that are sprayed onto CBB female founders during migration from refuges, at the peak of their flight activity, and onto fallen infested berries on the ground. An autoinoculation trap for CBB management with *B. bassiana* was proposed by Mota et al. (2017). A *B. bassiana* fungal strain was grown on a synthetic fabric that was incorporated in a trap baited with ethanol and methanol. The trapped CBB females are contaminated by the fungus before they leave the trap, and they act as reservoirs for pathogen dissemination in the crop. The autoinoculation trap provided high levels of CBB mortality in the field, but they attract a small portion of the pest (Mota et al., 2017). Small farmers in Brazil routinely use ethanol–methanol traps to monitor and to mass collect CBB (Silva et al., 2006; Fernandes et al., 2014). Thus, it is possible that using the *B. bassiana* bait trap would increase CBB control, but controlled experiments are necessary. Hollingsworth et al. (2020) showed the importance of using threshold-based *B. bassiana* sprays for CBB control in order to keep the control efficiency but with reduced costs as opposite calendar-based spray programs. Recently, Macedo et al. (2020) discussed the possibilities of disseminating *B. basssiana* spores by bees during coffee blooming and its contributition to the regulation of CBB populations. The main advantages of using *Beauveria* formulations are their low toxicity to workers and low impact on some beneficial insects (Mingotti Dias et al., 2020), but their high cost and variable efficiency should be considered. Finally, is worth to mention that the propagule viability and infection of *B. bassiana* on CBB are favored in coffee plantations under managed shade compared to full sun exposure, possibly due to the interception of solar radiation and higher humidity (Edgington et al., 2000; Turro et al., 2013; Mariño et al., 2016).

Organic Farming-Compatible Products

Among the products allowed in organic coffee production are sulfur-based and botanicals. Lime sulfur, a mixture of calcium polysulfides obtained by boiling calcium hydroxide and sulfur, has toxic effects on some insects and mites. The control of mites (*O. ilicis*) on coffee can be achieved by spraying lime sulfur at a concentration of 0.5% (Tuelher et al., 2014). Higher concentrations are unnecessary for mite control and should be

avoided due to deleterious effects on natural enemies, especially on phytoseiid mite predators (Venzon et al., 2013a; Tuelher et al., 2014). Although lime sulfur is used by some farmers aiming to control other pests, such as CLM, it has only ovicidal effect on this pest and at a higher concentration (>1.6%) and has no significant effect on CLM larvae mortality (Venzon et al., 2013b). No reported data about the control of scales on coffee with lime sulfur is available, but considering its efficiency in controlling these insects in other crops (Afonso et al., 2007; Venzon et al., 2016), we expect a negative effect on scale infestations.

Neem (*Azadiracta indica* A. Juss)-derived products are the most commonly used botanical pesticides in Brazil. Martinez and Meneguim (2003) reported a reduction on CLM oviposition when coffee seedlings were either treated with neem oil (0.125–2.5%) or with neem leaf extract (20–40%). Coffee seedlings sprayed with 0.1 g/L of azadirachtin did not prevent CLM female oviposition, but mine development stopped when leaves with eggs or larvae of CLM were treated with 0.025–0.1 g/L of azadirachtin (Venzon et al., 2005). The neem seed extract has a systemic and translaminar effect that permeated into the leaves, stopped the CLM development, drastically reduced the pupation, and prevented adult emergence (Venzon et al., 2005). Plants treated with neem products are expected to have a lower CLM infestation, either because treated plants repel ovipositing females or because CLM development is negatively affected by neem. For CBB, some laboratory studies show a different mortality rate (Depieri and Martinez, 2010). Concentrations of azadirachtin above 0.065 g/L reduced the population growth rate of *O. ilicis* (Venzon et al., 2005). By carefully choosing the formulation and concentration, based on research data and technical information, the side effects on natural enemies can be minimized (Depieri et al., 2005; Venzon et al., 2005). Several other plant extracts were tested and have promising results, under laboratory and/or greenhouse conditions, for CLM and CBB control (Santos et al., 2010; Alves et al., 2011; Fanela et al., 2020).

CULTURAL PRACTICES

The main cultural practices for pest control in coffee crops are related to CBB and consist of harvesting dry overripe fruit on trees and cleaning up of abscised fruits on the ground to reduce CBB reservoir in the inter-crop season (Aristizábal et al., 2016; Johnson et al., 2020). A bioeconomic analysis considering the multitude of factors that influence coffee production was performed by Cure et al. (2020). In their analysis, the authors used a system model that incorporates realistic field models based on considerable new field data and models for coffee plant growth, CBB development, and dynamics on CBB control strategies, including biological control. Their analysis estimated the potential of each CBB control tactic singly and in combination. Their conclusions, based on the analysis, were that the periodic harvest of fruit and the cleaning up were the major control practices that reduced the CBB infestation levels both in Colombia and in Brazil. They also added that the efficacy of the practice decreases as the time between harvests and cleanup increased from 15 to 60 days. It is important to point out that this cultural measure is more feasible in regions with one and short harvesting season, as in Brazil, than in places with two or long harvesting season, such as Colombia. In fact, this is a common practice in Brazil, especially adopted by small farmers, and it was intensified after some pesticide restriction to CBB control. At lower slopes in the Cerrado, Brazilian coffee farmers use a mechanized set for the collection of coffee berries that have fallen on the ground (Tavares et al., 2015; Alvarenga et al., 2018). Other post-harvesting control for CBB is the use of alcohol-based traps around the processing facility to capture any CBB that escapes from the processing facility (Aristizábal et al., 2016).

CONCLUDING REMARKS

Coffee agro-ecosystems are managed by human labor, which means that providing ecosystem service of biological pest control depends on a co-work between human and nature (Bengtsson, 2015). This revision reports a variety of strategies based on habitat manipulation for conservation biological control of coffee pests that have also the potential of conserving biodiversity. This approach has other benefits such as protection of soil from erosion, enhanced soil fertility and moisture, prevention of weed growth, carbon sequestration, and nutrient cycling. Plant diversification in coffee agro-ecosystems is also important for mitigating the effects of rising temperatures on coffee production due to climate change. The associated plants, being either fruit and wood trees, cover crops, or non-crop plants, have the potential of not only providing direct and indirect income to coffee farmer families but also aggregate value to coffee that will be produced with low external inputs and following regenerative practices. Production of such coffee will allow farmers to enter in specialty coffee marketing, a growing market where a high price for good-quality, high-biodiversity, and sustainable coffee is paid. There are knowledge gaps that need to be filled in the adoption of conservation biological control of coffee pests, but there are also plenty of opportunities to use the reported techniques and implement them for scaling regenerative coffee production. The importance of Brazil in global coffee production and the fact that it is the most biodiverse country in the world open up an opportunity for its prominence in the adoption of agrobiodiversity, fulfilling the dual role of agricultural production and environmental conservation. Finally, to achieve such goal, a collaborative work supported by public policies among farmers, researchers, field extensionists, industry, coffee traders, and consumers is necessary.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this publication and has approved it for publication.

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A Study of Regenerative Farming Practices and Sustainable Coffee of Ethnic Minorities Farmers in the Central Highlands of Vietnam

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Coffee is highly vulnerable to climate change, thus impacting coffee-dependent livelihoods and economies. As rising temperatures continue to reduce the suitability of many historical coffee-growing regions, some farmers are practicing regenerative, organic coffee farming as a means of climate change mitigation. In the Central Highlands, the primary coffee growing region of Vietnam, conventional sun-grown, monocrop coffee requires intensive inputs, including fertilizers, pesticides and water. However, some farmers are converting their conventional sun farms to organic shade farms utilizing regenerative farming techniques for both environmental and economic reasons. This study examined regenerative farming practices and sustainable coffee in a small ethnic minority village in Lâm Đồng province. The comparative analysis between soil samples taken from a regenerative shade-grown coffee farm and two conventional sun-grown coffee farms revealed that the soil of the regenerative farm, enriched with organic manure, is comparable to, or healthier than, the soil on the conventional farms enriched with chemical fertilizers. The results indicate that regenerative farming practices promote biodiversity; however, they also maintain microclimates that promote the growth of Roya fungus, which can decrease coffee yields. The economic analysis of farm costs and net returns found that regenerative farming practices decrease external inputs through a system of crop diversification and integrated livestock production that improves productivity and economic performance while preserving the ecological and environmental integrity of the landscape. Regenerative agriculture is an important step toward climate change adaptation and mitigation; however, in order for the farm communities in the Central Highlands to make the transition to regenerative agriculture, the success factors and benefits of this method must be demonstrated to the coffee farmers.

Keywords: regenerative agriculture, sustainability, coffee production, ethnic minority, Central Highlands, Vietnam

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INTRODUCTION

Globally, the livelihoods of more than 125 million people depend on coffee (Voora et al., 2019), the second-largest traded commodity after crude oil. Coffee is the second-most produced agricultural product in Vietnam, and the world's second-largest coffee producer after Brazil [International Coffee Organization (ICO), 2019]. Ever since the French first introduced coffee in 1857, generations of ethnic minorities in the Central Highlands of Vietnam have cultivated coffee as a reliable source of livelihood. Today, the production of coffee in Vietnam is 95% privately-owned, involving 640,000 small farms [International Coffee Organization (ICO), 2019], of which only one percent of coffee farmers own more than five hectares of land. Eighty-five percent of all farms are smaller than two hectares (World Bank, 2004), which generate an average yield of 2.3 tons per hectare, one of the highest in the world [International Coffee Organization (ICO), 2019].

In the Central Highlands, the areas of coffee cultivation are ∼583,000 hectares, accounting for 88% of the total cultivation area in the country [International Coffee Organization (ICO), 2019]. Sustainable coffee production has been certified by national and international organizations, such as VietGap, UTZ, Rainforest Alliance, and 4C. These farms account for more than 30% of the areas of cultivation [International Coffee Organization (ICO), 2019]. The coffee industry in Vietnam contributes significantly to the agricultural sector, in which share of coffee as percentage of agricultural GDP is about 12%. Vietnam's coffee export volume in recent years reaches about 1.5 million tons annually, with turnover of around USD 3 billion a year.

Climate change and related rising temperatures are changing the viability of farms in the Coffee Belt regions, creating less suitable environments for coffee cultivation and threatening the economies and livelihoods dependent on it (Bunn et al., 2015; Bejan et al., 2018). The primary coffee growing regions in Vietnam are located in five provinces collectively known as the Central Highlands, which historically have maintained ideal climates for *Coffea canephora* or Robusta coffee. Of all the coffee grown in Vietnam, 95% is Robusta and 5% is Arabica (Haggar and Schepp, 2011). The density for Robusta is about 1,330 trees per hectare, while the density for Arabica varies between 2,660 and 6,660 trees per hectare, depending on the variety and environmental conditions (Primecoffea, 2019). Historically, Robusta has been cultivated in Vietnam as a monocrop with high yields that require intensive fertilizers, pesticides and water. These monocrop cultivation practices have made coffee production in this region especially vulnerable to the projected impacts of climate change (Haggar and Schepp, 2011). The ethnic minorities who rely solely on coffee for their livelihoods suffer the most from climate change (Le et al., 2020). The better-off are those practicing sustainable intensification such as diversification of cropping systems and integration of livestock production systems as a means of adaption and mitigation to climate change.

The objective of this study is to examine regenerative farming practices and sustainable coffee in a small ethnic minority village in Lâm Đồng province of the Central Highlands. This study includes a comparative analysis of soil samples taken from a regenerative shade-grown coffee farm and two conventional sun-grown coffee farms, in addition to an economic analysis of farm costs and net returns.

BACKGROUND

Coffee and Climate Change

Research indicates that coffee is highly sensitive to the impacts of climate change. A multimodal database that uses machine learning algorithms to derive functions of global climatic suitability from geo-referenced production locations found that higher temperatures are predicted to reduce yields and suitable growing area for both Arabica and Robusta coffee (Bunn et al., 2015). For the top two producers of coffee in the world, Brazil and Vietnam, researchers predicted that rising temperatures may cause both countries to become unsuitable for coffee growth in the foreseeable future (Bunn et al., 2015). Although higher latitudes and altitudes with lower temperatures may maintain suitable growing conditions, migration to these areas could threaten ecosystems through deforestation (Läderach et al., 2013) and reduce producer resilience to climate change due to migration-related lack of available family labor (Baca et al., 2014).

Robusta coffee thrives in ideal temperature ranges of 20-30°C (Haggar and Schepp, 2012). However, 25% of global coffee growing regions currently reach temperatures higher than 30°C during the hottest month, and by 2050, this is projected to increase to 79% (World Coffee Research, 2017). These projected increases in temperature reduce suitability for coffee production, which will have major implications on coffee yields and coffee-dependent livelihoods in the future.

Since the 1960s, the average annual temperature in Vietnam has increased by 0.4°C, at a rate of about 0.09°C per decade, with the most rapid increase during the dry season between November and April (Haggar and Schepp, 2011; data from the World Bank's Climate Change Knowledge Portal, n.d.). Although overall temperature increases are occurring most rapidly in the southern regions, the central coffee growing regions have experienced significant warming. In the Central Highlands, the frequency of hot days and nights has increased every season since 1960, with the average number of hot days per year increasing by 29 days and the average number of hot nights increasing by 49 days (Haggar and Schepp, 2011). In contrast, the average number of cold days has decreased by 11 days and the average number of cold nights has decreased by 35 nights (Haggar and Schepp, 2011). These trends are detrimental to coffee production because increased hot days extend the period of unideal temperature conditions, which decreases coffee yields.

Water usage is another factor that makes coffee plants vulnerable to climate change. Climate variability may threaten future coffee production due to competition for water. In the Central Highlands region, evapotranspiration is predicted to increase by 8.5% between 2040 and 2059, and by 14.47% from 2080 to 2099 (Haggar and Schepp, 2011). Increased evapotranspiration will require increased irrigation to meet the water requirements necessary for healthy coffee plants, which increases environmental and economic costs.

Regenerative Agriculture and Shade Coffee as a Climate Change Mitigation Practice

Regenerative agriculture has five common goals according to Elevitch et al. (2018): "(1) Soil: contribute to building soils along with soil fertility and health; (2) Water: increase water percolation, water retention, and clean and safe water runoff; (3) Biodiversity: enhance and conserve biodiversity; (4) Ecosystem health: capacity for self-renewal and resiliency; (5) Carbon: sequester carbon."

According to a study by Regeneration International (2017), regenerative agriculture is a dynamic and holistic land management practice, incorporating organic farming and permaculture to improve soil health and fertility, thus increasing food production and income for farmers while preserving the environment. The framework of regenerative agriculture is divided into four different levels: "(1) functional; (2) integrative; (3) systematic; and (4) evolutionary." Level 1 focuses on reversing climate change through regenerating soils with best practices. Level 2 put an emphasis on integrative design and carbon farming to regenerate the ecosystem. Level 3 focuses on regenerating enterprise ecosystems with multi-capital flows and investments. Lastly, level 4 examines the regenerative cultural systems focusing on agriculture as ritual for the development of regenerative producer networks. Despite being multi-layered, each level is built upon the previous one, carrying the benefits onwards (Soloviev and Landua, 2016).

Sustainable coffee is an umbrella term that encompasses organic, fair and direct trade, eco-friendly, and shade-grown coffee production practices (Giovannucci and Koekoek, 2003). In principal, sustainable coffee ensures that production techniques and methods of distribution are enhancing the environmental, economic, and social well-being of all agents involved. Environmental sustainability represents the environment and ecological conditions of the farm; social sustainability describes the production system that maintains respect for social principles that are benefiting the community; and economic sustainability allows the farming management to be financially viable. Regenerative agriculture is an integral part of sustainable coffee. According to a previous study "there is now strong evidence that regenerative and resource conserving technologies and practices can bring both environmental and economic benefits to farmers, communities, and nations," (Pretty, 1995, p. 1).

As climate change continues to reduce the suitability of coffee growing regions, human adaptation methods are necessary to reduce the vulnerability of coffee-based communities and economies. The incorporation of shade trees in coffee farms is one mitigation technique that encourages higher biodiversity, as shade trees provide habitat for diverse species and serve as carbon sinks. Studies within the last 25 years indicate that shaded coffee farms promote high biodiversity of invertebrates, vertebrates, and plants, which help maintain the health of coffee agroecosystems (Perfecto et al., 2007). Shade-grown coffee can be classified into different shade levels and management according to a study in Mexico by Moguel and Toledo (1996, 1998), and by Gobbi (2000) in El Salvador. The classification is as follows: rustic (coffee is grown under the shade of a natural forest); traditional polyculture (similar to the rustic system in structure, but has a greater diversity of economically valuable shade trees planted by the farmer); commercial polyculture (shade trees are mostly planted as alternative commercial products); and technified shade (original forest has been completely removed and replaced with a few shade tree species). Most coffee farmers practice commercial polyculture by growing timber and fruit trees adaptive to the region because of the additional monetary benefits they provided (Albertin and Nair, 2004). Shade trees can also serve to regulate the stability of annual coffee yields (Albertin and Nair, 2004). The authors explained that instead of having an unpredicted low and high yield harvests under sun farms, shade farms have more stable annual yields.

Because shaded coffee systems promote biodiversity, they provide valuable ecosystem services that maintain complex invertebrate interaction webs, resulting in autonomous pest control (Vandermeer et al., 2010). In addition to promoting the presence of natural pest regulators, shaded systems promote higher diversity of native pollinators which contribute to higher coffee yields (Perfecto et al., 2007). Previous studies indicate that shaded coffee systems also help mitigate extreme temperature and precipitation changes, with high potential as an adaptation technique for projected climate change. The canopy cover of shade trees protects coffee plants from harsh sunlight and reduces temperatures by around 4°C (Läderach et al., 2013), which may improve the resilience of coffee plants in warmer regions. Shade canopies also improve soil moisture retention and protect coffee plants against erosion and landslides (Läderach et al., 2013).

In addition, shade trees increase bird habitat and abundance on coffee farms, which reduces coffee pests. Johnson et al. (2010) found that the presence of birds reduced the amount of coffee berry borer, *Hypothenemus hampei*, which is the most damaging coffee pest. The study also noted that the reduction of the borer pest by birds on coffee farms resulted in cost savings, illustrating the economic advantages of shade trees on coffee farms. As a natural form of pest prevention, shade-grown coffee farmers with increased bird abundance can reduce their pesticide input, thus reducing input costs and environmental damage. An additional study conducted in India found that reduced pesticide use in Robusta agroforests could lead to increased food resources for birds, thus improving biodiversity (Chang et al., 2018).

Although these benefits of shaded coffee production mitigate impacts of climate change, lower yields as a result of climate pressures have led to a shift toward coffee monoculture in traditionally shaded areas, reducing climate change resilience (Läderach et al., 2013). In comparison to shaded farms, the resilience of monoculture coffee farms to erosion, increased evapotranspiration, drought, and extreme weather events is very low (Haggar and Schepp, 2011).

Constraints of Sustainable Coffee Production

Sustainable coffee has clear benefits for farms and farming communities; however, there are disadvantages to certain methods of sustainable production. First, the initial transition

of a conventional farm to an organic or shade-grown farm can be labor intensive, expensive, and take several years to establish. The introduction of shade trees and other shade crops can have initial costs that require farmers to take out loans or find other means to finance the transition. Second, in order to obtain certifications such as organic or fair trade, farmers need to finance the certification organization to assess their farm, fill out detailed paperwork, and then address any requirements needed to become officially certified (Guthman, 2004). Certifications such as organic require farms to produce for 3 years without any pesticides or herbicides before officially being certified (Riddle, 2003). This can impact the economic viability of farms as they strive to transition, but lack the price premiums of organic sales initially (Le et al., 2017). Furthermore, farmers will need to improve productivity to maintain or increase their income as the sustainable coffee market will reach its maturity and price premiums will be expected to decrease (Kilian et al., 2006). Lastly, certifications have become a norm in some regions, forcing farmers to obtain certifications or not be able to sell their beans to exporters without them (Le and Jovanovic, 2019).

In terms of the farm itself, the initial transition from conventional to organic farming can increase susceptibility to various pests and diseases (Bengtsson et al., 2005). In addition, shade trees have shown increases in fungal attacks, specifically by the fungus *Mycena citricolor,* due to an increase in humidity on the farm (Beer, 1987). Furthermore, over-shading can reduce coffee yields significantly. For example, Soto-Pinto et al. (2000) found that shade cover above 50% can significantly limit coffee yields, while 30–45% shade cover promoted the highest yields. Another study found that competition for water between coffee plants and shade trees may reduce coffee health during the dry season (van der Vossen, 2005). Shade trees can also damage coffee plants with falling branches, and the need to prune shade trees regularly poses additional labor costs.

Roya Fungus in Shaded and Sun Agricultural Systems

The Roya fungus, also known as coffee leaf rust, *Hemileia vastatrix*, is one of coffee's largest pests and is believed to have originated in Africa (Kushalappa and Eskes, 1989; and McCook, 2006). Records indicate that rust first devastated coffee crops in Sri Lanka in the middle of the 19th century (Vandermeer et al., 2010). In 1970, the disease spread to Brazil, and later to both Central America and the Caribbean (Vandermeer et al., 2010). Between 1865 and 1985, the rust disease spread globally to all the coffee growing regions with varied degrees of biological and economic impact (van der Vossen, 2005). Rust primarily attacks the leaves of coffee plants, leaving yellow-orange spots usually 2–4 millimeters in diameter. Over time, the spots brown and eventually become necrotic; the diseased leaves fall prematurely (Kushalappa and Eskes, 1989). Severe cases of rust can cause delayed growth, defoliation of the entire plant, premature berry dropping, and can slowly kill the coffee plant (Kushalappa and Eskes, 1989).

The impact of shade management techniques on the presence of the Roya fungus is not currently well-known. Some studies have indicated that shade management practices can reduce the risk of rust epidemics while others indicate intensification of coffee rust in shaded systems (Avelino et al., 2004). These inconsistencies are likely a result of the multifaceted effects shade has on coffee plants. Shade can reduce fruit loads, which decreases coffee leaf receptivity to Roya fungus; however, shaded systems can also cultivate suitable microclimates for the germination and colonization of the fungus (López-Bravo et al., 2012). A study that dissociated these factors by homogenizing fruit loads on shaded and full sun coffee plants found that shade does have negative effects on the presence of rust because the intensity of rust was greater in the shaded systems when both systems had equal fruit loads. The reduction of fruit loads associated with shaded plants mitigates the intensity of rust in shaded systems, but is accompanied by the disservice of reduced coffee yields (López-Bravo et al., 2012).

THE STUDY REGION

The study site was located in Lăng Cú village, Gung Ré commune, Di Linh district. Di Linh district is located in Lâm Đồng province in the Central Highlands of Vietnam with a total area of 1,628 km^2. Gung Ré commune has an area of 121.5 km^2 with a population under 9,000 people and a population density of 69 people/km^2 (Lam Dong Portal, n.d.). Lâm Đồng province is characterized by its varied terrain with mountains and highland terrains in the north and hills and valleys in the south. Elevation in the northern districts of Lâm Đồng is around 1,500 meters, while the southern districts reach elevations of about 800–1,000 m. The elevation change in the Lâm Đồng province naturally separates the growth of various perennial crops based on their climate preferences. The cooler, high elevation climate of the north is ideal for the growth of high-quality Arabica coffee, while the hotter, lowlands in the south are home to the hardier Robusta coffee. Di Linh district is the largest producer of Robusta coffee in Lâm Đồng Province, with about 41,000 hectares of coffee cultivated throughout the region, accounting for 75% of agricultural land in the district. In the last 10 years, more than 50% of coffee plants have been replanted with new varieties. In Lăng Cú village, about 80 out of 330.7 hectares of coffee (∼25% of the farmland) have been replanted in recent years.

To conduct a comparative analysis between regenerative shade-grown coffee farming practices and conventional sun-grown coffee farming practices, we collected soil samples, conducted invertebrate counts, and compared the presence of the Roya fungus on three farms: one regenerative shade farm and two conventional sun farms as shown in **Figure 1**. An economic analysis of these farms was also conducted.

The K'Ho Perceptions of Sustainable and Conventional Coffee Cultivation in the Region

Part of this study included a survey of 30 ethnic minority K'Ho farmers (17 were female and 13 were male) who grow Robusta coffee in this village (Jovanovic and Le, 2019; Le et al., 2020). Twenty-two of the 30 farmers migrated over 35 years ago from

FIGURE 1 | Lăng Cú village, Gung Ré commune in Di Linh district of Lâm ồng province. Source: Google Earth, Image @2019 DigitalGlobe.

the mountainous area of Son Điền commune to Lăng Cú village, where the local government initially gave each family 100 coffee trees and between 4,000 and 5,000 m^2 of land. Some families expanded their land up to 3–4 hectares until the government restricted land expansion around 15 years ago. These farmers first cultivated dry rice on the hillsides, then wet rice in the flatlands, and finally switched to coffee cultivation on the hillsides around 25 years ago. Today, each family has an average of 2–3 hectares of coffee farm and 1 hectare of wet rice field. A coffee-rice segregated system is typically chosen by farmers in the Central Highlands as a form of subsistence for food security because rice is a stable crop (Ho et al., 2017). Each hectare of conventional sun-grown coffee yielded an average of 2.5 tons, earning a net income of 20 mil VND ($870) per ton, compared to 1.5 tons shade-grown organic coffee with an expected earnings of 67 mil VND ($2900) per ton (Le et al., 2020).

Currently, the village has 185 households with 764 people, of which the K'Ho accounts for 40.5% (Lam Dong Online, n.d.). The history of chemical use in the village began with the initial migration to the province. The government gave farmers fertilizers and pesticides for rice cultivation upon arrival to the region. Although the government subsidies of chemicals ended between 6 and 7 years ago, most farmers in the area continue to use chemicals on both their rice and coffee farms. The farmers indicated that the quality of their soil is progressively worsening, decreasing coffee yields; they use chemicals to counteract the infertile land, but also attribute lower soil quality to a history of chemical use.

The average age of the 30 farmers surveyed is 52 years old, the youngest is in the late twenties and the oldest farmer is over 80 years old. The average size of the family is 8. Out of the 30 farmers, only three farmers cultivate sustainable shade-grown organic coffee. The farmers that practice organic cultivation are young, college graduates who have perceived negative impacts of chemical fertilizers and pesticides and want to practice more sustainable soil management. Shade-grown organic coffee is viewed as an experimental farming practice in this village and other farmers are waiting to see how well the organic coffee succeeds environmentally and financially before they consider implementing the technique. Despite a higher projected net

income from organic coffee, only young farmers are considering transitioning their farms because the initial expenses and labor costs of conversion are considerably high for the older farmers. Many farmers are also concerned that without pesticides, their crops will be overrun by pests in the area, particularly the borer beetle and Roya fungus (Le et al., 2020).

Farmers in the village have already perceived impacts of climate change on their coffee production. They have noted that increases in heatwaves, periods of intense rainfall and drier, nutrient-deficient soil have all lead to recent declines in production. Farmers are already grappling with decreasing harvests due to climate change; therefore, despite research indications that switching to more sustainable cultivation methods may mitigate the impacts of climate change, farmers are concerned that removing pesticides and chemical fertilizers will lower their production even further. However, research has shown that regenerative farming practices that focus on restoring soil health actually increase farm productivity (Sherwood and Uphoff, 2000).

Study Area 1: Shade Farm (Regenerative Agriculture)

The first research site was a regenerative shade-grown coffee farm, started by a K'Ho farmer who graduated with a degree in soil management and is a leader in his community in the regenerative farming movement. This farmer learned regenerative farming practices in college and integrated these techniques into his own coffee farm after graduating in 2013. His family owns a total of four hectares of land, of which three hectares are allocated for coffee production and one hectare for wet rice cultivation. The three hectares for coffee production are divided into smaller plots along the hillsides of the village. Currently, the farmer in this study has been implementing functional regenerative agriculture (level 1) and integrative regenerative agriculture (level 2) on his farm. In level 1, the farmer's objective is to regenerate the soil that has been damaged from years of chemical use. To complete level 2, he must improve the health and strength of the whole farm ecosystem through the integration of shade tree species and livestock for optimal biodiversity and carbon capture.

The farm revealed in **Figure 2** is an experimental farm with a total area of 5,000 m^2. 1,000 m^2 contain the farmer's main house, a traditional wooden house, animal cages, an area to dry coffee, and a fertilizer storage area. To the southeast, the farm is located next to one hectare of wet rice fields owned by the same farmer. The remaining 4,000 m^2 (in a triangular shape) comprise the shade coffee farm classified as a commercial polyculture system, consisting of 450 coffee trees and other tree species planted in 2014. Dispersed among the coffee plants, the farmer planted 200 *Senna siamea* trees to shade the coffee plants. He chose *Senna siamea* because this particular plant has a rapid growth rate and attracts birds that eat coffee pests. The farmer explained that there is no need for pesticides to treat the coffee pests. The birds eat the coffee berry borer once migrated to the *Senna siamea* trees. Additional benefits of these trees include the ability to use the leaves as an organic green compost, stabilizing soil properties of tree roots, and that the roots of the trees provide micro-organisms that keep the soil fertile and provide nutrients to the coffee plants. Third, these trees also contain economic value because after 5 years, they can be harvested for timber.

In developing countries, the diversification of cropping systems does not only depend on cash crops, but also on food crops for household consumption (Scialabba and Müller-Lindenlauf, 2010). Zhang and Li (2003) demonstrated that intercropping utilizes soil nutrients more efficiently, thus increasing productivity. Crop diversification is an important factor on this experimental farm. In addition to *Senna siamea*, tree varieties on the farm include jackfruit, guava and banana. Between 2017 and 2018, the farmer also planted 70 macadamia trees that were provided by the local government as an experiment to see if macadamia can thrive in the elevation and climate of this region as a possible alternative commercial product. On the ground, the farmer also grows different types of vegetables and mushrooms for consumption. All these plant species feed the family and animals, and leftovers can be sold to the local and farmer markets. Animals on the farm include three cows, 12 goats, one black pig (native to this region), and chickens. The farmer recently sold dozens of pigs and used the money to build larger cages for the next pig herd. The cows and goats eat *Pennisetum purpureum* grass and straws from the rice fields. The pigs eat banana leaves and trunks. The livestock on the farm provide multiple benefits to the household and are important assets for the family, as major food sources and as manure sources for organic fertilizer.

The farmer does not use any chemical fertilizers on his farm; rather he makes his own organic fertilizer collected from cow, goat and pig manure. He creates a mix of 80% manure and 20% rice husks and straws; he learned this optimal ratio from a workshop organized for farmers in the community by the Japan International Cooperation Agency (JICA). He combines the mixture with yeasts from a traditional K'Ho wine and stores the mixture under a plastic cover for a fermentation period of 2–3 months. The farmer only applies organic fertilizer and refrains from all chemical pesticide usage on his farm. In contrast, other farmers in the village use chemical fertilizers which they mix with animal manure and store for only 1 month. All of the farmers in the village apply fertilizer to their coffee trees three times between March and June: the first time in March before the rainy season, the second time in May during the rainy season, and the third time in late June after the rainy season.

The sustainable farming system implemented by the farmer on his experimental farm integrated coffee with other crops (*Senna siamea* and fruit trees) and rice has proven to be more efficient than the current conventional systems (sun-grown coffee mono crop and sun-grown coffee and rice crops) practiced by the majority of the famers in the village. This is consistent with the findings in coffee farming communities reported in Ho et al. (2017). Furthermore, the regenerative farming practices limited the use of external inputs through a system of integrated livestock production to improve productivity and economic performance while preserving the ecological and environmental integrity of the landscape. Regenerative agriculture levels 1 and 2 (Soloviev and Landua, 2016) have demonstrated that these are important

FIGURE 2 | Triangular shape shade-grown coffee farm (regenerative) in 2019 vs. 2013. Source: Google Earth, Image @2013 and @2019 DigitalGlobe.

steps toward climate change adaptation and mitigation for sustainable intensification as described in Campbell et al. (2014).

Study Area 2: Sun Farm A

The second farm site is a conventional sun-grown coffee farm located directly adjacent to the shade farm. This is a typical unshaded monoculture system in the Central Highlands of Vietnam. Under this system coffee trees are exposed to full sun and are highly reliant on chemical inputs and water, which make them financially vulnerable to market fluctuations (Gobbi, 2000). The farmer of the shade farm waters his farm two times per year during flowerings, whereas the sun farms are constantly being watered, especially during the hottest months. The analysis was conducted on a 2,000 m^2 plot containing about 400 coffee trees sharing a property line with the shade farm. The coffee plants on this farm are over 25 years old and very low in productivity. Chemical fertilizers and pesticides are used on this farm. Soil erosion and degradation are apparent on this sun farm. The rice fields have been slightly impacted by erosion and soil running down hills into the lowlands; however, there is little impact on the shade farm and the rice fields located right below it.

Figure 3 provides an image of the shade farm and sun farm for comparison. The shade farm on the left is greener and has more biodiversity compared to the sun farm on the right. There were no birds and far fewer ants and other invertebrates on the sun farms. This observation is consistent with a previous study in Latin America coffee plantations (Philpott et al., 2008), which revealed that sun coffee exhibits the highest losses of ant and bird species, and these losses increase with management intensity. Since coffee trees require light to produce and for the cherries to mature, light shade is preferred over thick shade. **Figure 4** provides an image of the coffee-*Senna siamea* intercropping with spreading crown to allow light to filter onto the coffee trees. The

FIGURE 3 | Shade farm and sun farm A. Source: Photo provided by the owner of the shade farm. Shade farm covered with *Senna siamea* trees is on the left. Sun farm A is on the right.

shade farm had a much cooler temperature than the sun farm which was around 27°C on the field research days in March.

Study Area 3: Sun Farm B

The third farm site is a sun farm owned by the family of the same farmer who cultivates the shade farm. Chemical fertilizers and pesticides are used on this farm. This farm is located on a hill across from the shade farm, separated by wet rice fields. The total area of the sun coffee farm is 2,000 m^2, containing over 200 coffee trees that are over 25 years old and also very low in productivity due to old age. Most of the coffee trees on this farm are infested with *Phytophthora*, a white fungus which harms coffee plant trunks, branches, and bark. The farmers interviewed noted that most of their trees have an apparent presence of this water mold. This water mold decreases the productivity of their coffee trees. In order to combat the mold, some farmers have applied *Trichoderma harzianum,* a natural fungicide that attacks the mold. This is an example of a natural method of farm management that is being applied to minimize the use of chemical pesticides.

METHODS AND RESULTS

Site Designation and Soil Sampling

The USDA's Soil Quality Test Kit Guide and Soil Quality Indicator Sheets were referenced to establishing the soil sampling method used in this study. Due to limited time and resources, one round of soil measurements was collected to compare to standard soil conditions instead of multiple soil samples taken over an extended period of time. On each of the three farms (shade farm, sun farm A, and sun farm B) five sites labeled A–E were designated on the north, south, east, west, and middle of each farm. This methodology was used instead of random sampling on each farm to ensure that soil near the border of neighboring farms was collected in case neighbor's farms were impacting the soil health of each farm. At each of the five sites, four soil samples were taken using a soil corer drilling 0.5 m below the surface, for a total of 20 samples taken at each farm. The top 0.25 m of topsoil was removed and the bottom 0.25 m of soil was stored in sealed plastic bags. The samples from each farm were transferred to the Da Lat Nuclear Research Institute for analysis. The soil samples were analyzed between one and 2 days after retrieval from the farms. Soil samples weighing 0.5 kg were analyzed according to dry weight at 105°C. Total nitrogen, total phosphorus, total potassium, bulk density, and pH were calculated by the research institute. **Tables 1**, **2** present the paired samples *t*-test for comparison of the mean scores of the soil samples on the farms.

A *t*-test was calculated to determine if there was a statistically significant difference between the soil components of the shade and sun grown coffee. The result revealed that there is a significant statistical difference in pH at the 10% level between the shade farm and sun farm A and sun farm B, indicating that the pH of the soil from the shade farm was statistically higher than the pH of the soil in both sun farms. According to a study on land use requirements for Robusta coffee, the optimal pH ranges

FIGURE 4 | Shade farm—Coffee-*Senna Siamea* intercropping. Source: Photo provided by the owner of the shade farm.

TABLE 1 | *T*-test of soil analysis in shade farm vs. sun farm A.

	Shade farm	Std. Dev.	Sun farm A	Std. Dev.	t-stat.
pH (KCl)	4.146	0.510	3.726	0.072	1.946*
Total nitrogen (ppm)	1888.400	327.291	1942.000	211.463	−0.308
Total phosphorus (ppm)	949.400	357.250	1142.000	434.645	−0.765
Total potassium (ppm)	231.800	57.454	143.160	65.013	2.284**
Bulk density (g/cm^3)	1.605	0.015	1.602	0.014	0.257

*Sig. 2-tailed. **significant at 5% level; *significant at 10% level; equal variances assumed.*

TABLE 2 | *T*-test of soil analysis in shade farm vs. sun farm B.

	Shade	Std. Dev.	Sun farm B	Std. Dev.	t-stat.
pH (KCl)	4.146	0.510	3.702	0.030	1.944*
Total nitrogen (ppm)	1888.400	327.291	1846.200	317.807	0.207
Total phosphorus (ppm)	949.400	357.250	834.600	242.551	0.594
Total potassium (ppm)	231.800	57.454	142.380	37.702	2.910**
Bulk density (g/cm^3)	1.605	0.015	1.603	0.016	0.141

*Sig. 2-tailed. **significant at 5% level; *significant at 10% level; equal variances assumed.*

between 5.0 and 6.3 KCl, but possible extremes can range from 4.0 to 8.0 KCl (Haggar and Schepp, 2012). The average pH of the shade farm (4.146 KCl) was closer to the optimal pH range conditions for Robusta coffee than the average pH of the sun farm A (3.726 KCl) and sun farm B (3.702 KCl), respectively.

There is also a statistically significant difference at the 5% level between the soil samples of the shade and sun farms in the total amount of potassium, which is beneficial for fruit development and higher yields. Potassium level at the shade farm was higher on average (231.8 ppm) compared to sun farm A (143.16 ppm) and sun farm B (142.38 ppm), respectively. Higher potassium levels of the shade farm were unexpected when compared to previous studies, which found that many organic systems need to add significant quantities of additional composted organic matter from external sources to meet nutrient demand, and that many organic farmers face lower yields because they are unable to acquire the additional compost (van der Vossen, 2005).

A study in El Salvador found that higher shade tree densities were associated with lower nitrogen and potassium levels possibly due to competition between the shade trees and coffee plants (Méndez et al., 2009). Shade tree densities are typically high in commercial polyculture system compared to very high in traditional polyculture system and medium in shaded monoculture (van Rikxoort et al., 2014). Contrary to this pattern of lower nitrogen levels on shade farms, there is a statistically insignificant difference found between nitrogen levels on the organic and conventional farms in this study. Nitrogen is a key factor impacting vegetative growth and coffee yields, which is usually 20–40% lower on organic farms when compared to conventional farms (van der Vossen, 2005). Previous studies indicated that organic farming systems usually fail to achieve optimal levels of available nitrogen exclusively through organic compost and manure (van der Vossen, 2005); however, the shaded system in this study did manage to supply the same amount of nitrogen as both sun farms that utilized chemical fertilizer despite the fact that *Senna siamea* trees are not nitrogen-fixing trees. The lack of statistical difference between nitrogen levels across organic and conventional farms indicates that the effectivity of organic compost in this study was comparable with that of chemical fertilizer.

Although the amount of phosphorus required in coffee plants is relatively small compared to other nutrients, it is a necessary macronutrient for root growth, fruit development, and cherry maturity. A lack of phosphorus poses a constraint to coffee yields. Coffee's main sources of organic phosphorus are leaves and pruning remnants (Ling et al., 1990). Additionally, organic or inorganic phosphorus (bone meal or rock phosphate) can be applied to cultivated soils. Another study found that the distribution of organic and inorganic phosphorus pools varied depending on the inherent characteristic of the agroecosystem, i.e., agroforestry vs. full sun (Xavier et al., 2010). In this study, there is no statistically significant difference in the levels of phosphorus between the shade and sun farms. The leaves from the coffee trees and *Senna siamea* trees provide nutritional efficiency in the shade farm without further application of phosphorus.

There is no statistically significant difference in the bulk density levels between the shade and sun farms. This was unexpected because bulk density is generally lower when soils are rich in organic matter and have higher porosity (USDA Natural Resources Conservation Service, 2008). It was anticipated that the regenerative farm would have lower bulk density than the other farms given the state of the soil as looser and richer in organic matter from the use of organic compost and shade cover.

Through visual observation, it was noted that the coffee plants on both sun farms were deficient in calcium, whereas the shaded plants were not. This observation is consistent with a study comparing soil properties of organic and conventional coffee systems that found organic systems contained significantly higher levels of carbon (Velmourougane, 2016).

Invertebrate Biodiversity Sampling

Following the same site designation as the soil sampling method, a 0.5 × 0.5 m quadrat was placed on the ground at each of the five sites A–E. The leaf litter inside the quadrant was sorted through and the number of individuals of each invertebrate taxonomic group was recorded to assess invertebrate abundance and biodiversity. This method was repeated at each of the five locations at each of the three farms. The data are presented in **Table 3**. The shade farm had greater invertebrate abundance and species biodiversity than both sun farms, which is consistent with previous studies (Perfecto et al., 2007; Vandermeer et al., 2010). These results indicate that the cultivation of shade coffee is less damaging to biodiversity than the cultivation of sun coffee.

Assessment of Roya Fungus Presence

Following the same site designation, A-E, one coffee plant was randomly selected at each of the five locations on each farm, and five branches on the chosen trees were randomly selected to inspect the leaves. All the leaves on the selected branches

TABLE 3 | Invertebrate abundance and biodiversity comparison.

	Shade farm	Sun farm A	Sun farm B
Total abundance	47	29	21
Total no. of species	5	4	3

The total number of invertebrates (abundance) and number of different species were counted inside of a 0.5 × 0.5 m quadrat at five locations across each farm.

TABLE 4 | Comparison of Roya fungus presence.

	Shade farm	Sun farm A	Sun farm B
No. of leaves inspected	432	436	370
No. of leaves infected	75	38	48
Percentage of infected leaves	17.36%	8.72%	12.97%

were examined for the presence of Roya fungus, and the total number of infected leaves on each branch was recorded and presented in **Table 4**. The shade farm had a higher percentage of coffee leaves infected with the Roya fungus, with 17.36% of leaves infected on the shade farm compared to 8.72% and 12.97% of leaves infected on the trees of sun farms A and B, respectively. While lower levels of fruit loads on the shaded coffee plants may decrease leaf receptivity to Roya, the higher resilience to rust of the shade plants did not outweigh the negative impact of the shaded microclimate that promotes the germination and spread of the fungus. These results are consistent with established literature on rust, because the shaded area cultivates a more ideal microclimate for the disease to cultivate and spread (López-Bravo et al., 2012).

Economic Analysis of Regenerative vs. Conventional Farming

This section pertains to the economic analysis of regenerative farming and conventional farming practices. A previous study by the U.S. Department of Agriculture (USDA) indicated that farmers who apply soil conservation methods and reduce their dependence on fertilizers and pesticides generally report lower production costs in comparison to conventional farms (Reganold et al., 1990, p. 112). In terms of output, organic agricultural systems in developing countries generate equal or even higher yields compared to conventional practices (Scialabba and Müller-Lindenlauf, 2010). With respect to coffee production, sustainable production using coffee husk compost to supplement cow manure and chemical fertilizers increases coffee yields in Vietnam (Nguyen et al., 2013).

Following the comparative analysis between sustainable and conventional farms described in Reganold et al. (1990), this study compares the costs and returns between the regenerative shade farm (commercial polyculture) and the conventional sun farm (unshaded monoculture) for the coffee harvest season 2018–2019 based on 4,000 m^2 of land in each farm. The results are shown in **Figure 5**. Despite higher total input costs incurred in the shade farm due to fixed costs, total cash income is significantly higher because of an improvement in the quality of coffee and a higher premium for organic, and additional income from other sources on the farm. Fixed costs include building infrastructure for the regenerative farm (i.e., shade trees, cages and livestock). Over the past 5 years, the farmer of the shade farm has invested about VND 100 mil. in building infrastructure (equivalent to USD 4,350). In 2018–2019 he invested VND 20 mil. in fixed costs. Currently, the variable costs for both farms are the same. However, in the future, it is expected that variable costs of the shade farm will be lower when compared to the sun farm when less purchased inputs are required. Variable costs for the shade farm include water, fuel for water irrigation, maintenance, straws and rice husks, yeasts for mixing with manure to make organic fertilizer, grass to feed the cows and goats, and labor for harvesting cherries. Variable costs for the sun farm include water, fuel for water irrigation, maintenance, chemical fertilizers, pesticides, and labor for harvesting cherries. Overall, the net returns are higher for the shade farm compared to the sun farm, being VND 42 mil (USD 1,826) vs. VND 12 mil (USD 522). The shade farmer expects his farm to generate higher net returns in the future when fixed costs are no longer required (or minimized) once the regenerative infrastructure is self-sustained and variable costs are lower.

The results of this analysis are similar to the findings of Reganold et al. (1990), which concluded that low-input sustainable agriculture reduces reliance on fertilizers, pesticides, and other purchased inputs. As a result, profits for the sustainable farms are higher. In addition, total cash income will also increase with the commercial polyculture system. The shade trees, *Senna siamea*, can be harvested for timber, and macadamia nuts have a high commercial value. To generate additional income, the farmer also sells the pig herd (when they reach 15 kg at 100,000 VND/kg) and the grass-fed goats to local markets. He is planning to commercialize the traditional K'Ho smoked pork for higher value-added. He is also in the process of creating a brand for his coffee with a trademark and will eventually roast the coffee with a purchase of a roaster. This would require an additional capital investment added to the fixed costs, but with high net returns in the long run.

A study in El Salvador reveals that investing in a commercial polyculture farm is profitable but also has the highest risk since coffee yields are lower (Gobbi, 2000). This is due to the farm's devotion to other economic activities, especially the planting of shade trees. Thus, regenerate agriculture must provide the potential for famers to realize viable livelihood as demonstrated in coffee micro-mills in Costa Rica (Nuñez-Solis et al., 2021). As an experimental farm, the farmer in this study seems to engage in multiple activities without taking the opportunity costs into consideration. In the future, as he plans to replicate and expand the regenerative farming practices to the rest of his family's farm, he has to find an optimal solution for coffee-shade tree-livestock interaction to sustain a biodiverse farm as well as his family livelihood.

CONCLUSION

The results of this study indicate that the soil health of regenerative shade coffee is comparable to or better than that of fertilized sun coffee. With higher and statistically significant

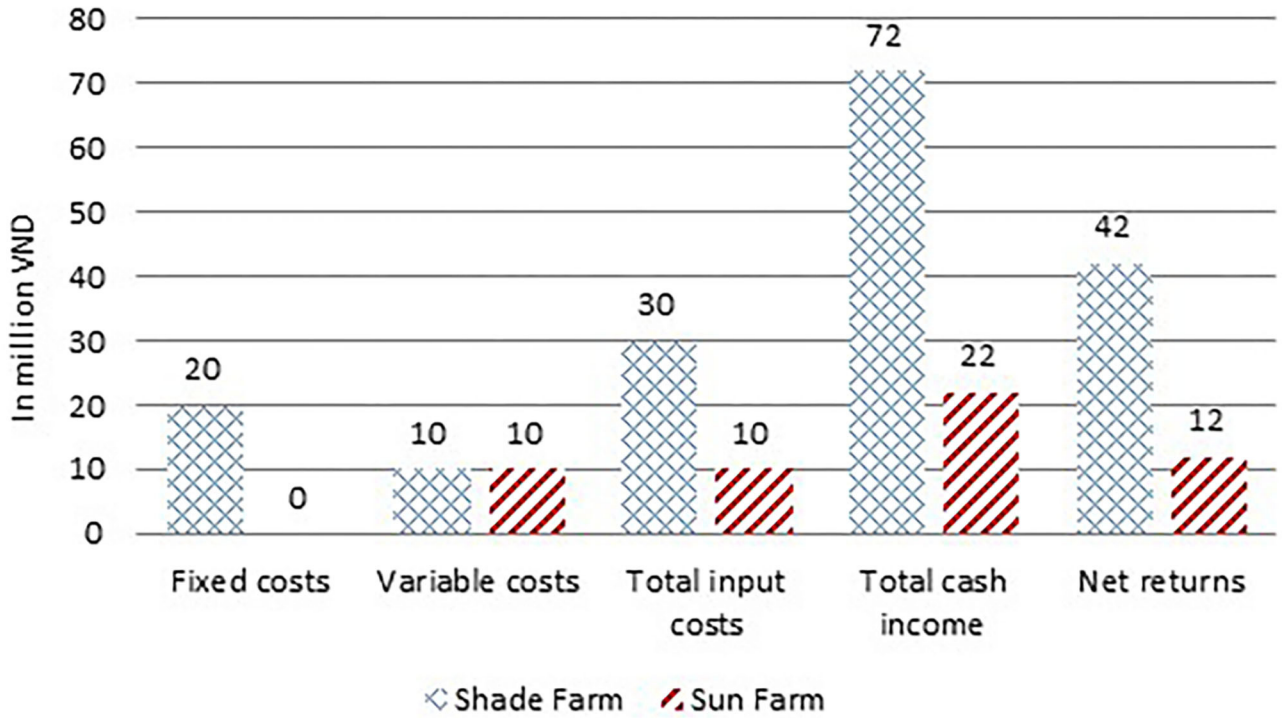

FIGURE 5 | Comparative costs and returns between shade farm vs. sun farm 2018–2019 Harvest. Exchange rate: VND 23,000 = USD 1. Based on 4,000 m^2 of land in each farm. Net returns = Total cash income – Total input costs. Total input costs = Fixed costs + Variable Costs.

pH and potassium levels on the shade farm, and statistically insignificant differences in the levels of nitrogen, phosphorus, and bulk density between the organic shade soil and inorganic sun soil, our results illustrate that organic compost can be just as effective as chemical fertilizers when supplementing soil health on coffee farms. Limiting the use of chemical fertilizers reduces input costs without compromising soil quality, resulting in higher profits for low-input, sustainably cultivated coffee. The shade farm had significantly higher levels of pH (at the 10% level) and potassium (at the 5% level) in comparison to the sun farms, indicating that the regenerative method resulted in more optimal pH and potassium levels for coffee cultivation. These factors may benefit vegetative growth, fruit development, and yield for coffee plants on shade farms.

Consistent with existing literature on shaded management systems and biodiversity, the shade farm exhibited greater species biodiversity than both sun farms. With greater species biodiversity and invertebrate abundance, our research indicates that the regenerative shade cultivation method is less damaging to biodiversity than the cultivation of sun coffee. The enhanced biodiversity of the shade farm likely benefits coffee production by maintaining the health of the coffee agroecosystem, encouraging natural pest control webs, and promoting a variety of pollinators, which increases coffee yields.

Given the ecological and economic benefits of regenerative shade coffee cultivation, we recommend sustainable, shaded coffee production as a viable adaptation method to mitigate the harmful effects of climate change in coffee growing regions of the Central Highlands of Vietnam. Higher net returns on shade coffee can improve livelihoods of coffee farmers economically, while ecologically protecting biodiversity and reducing climate change vulnerability. The future success of the experimental farm using regenerative agriculture at levels 1 and 2 is very important as many of the farmers in Lăng Cú village are waiting to see the results before deciding whether to alter their farm management techniques. The systemic regenerative agriculture (Level 3) can be achieved if the whole village begins gradually transitioning from conventional to regenerative farming practices. However, given a limitation of small soil samples collected on three farms in the village, this study can only be considered as a baseline study for further replication and investigation throughout this farm community to draw best practices at different levels of regenerative agriculture, and perhaps if successful, the model should be expanded to other farm communities in the Central Highlands. To make this effort feasible, it would require institutional support from the local government.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

QL and GJ: conceptualization. QL, SC, and GJ: methodology and writing—review and editing. QL, GJ, SC, and D-TL: investigation and data collection. QL: formal analysis, writing—original draft preparation, and supervision. D-TL and QL: visualization. All authors have read and agreed to the published version of the manuscript.

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Hot Coffee: The Identity, Climate Profiles, Agronomy, and Beverage Characteristics of *Coffea racemosa* and *C. zanguebariae*

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Climate change poses a considerable challenge for coffee farming, due to increasing temperatures, worsening weather perturbations, and shifts in the quantity and timing of precipitation. Of the actions required for ensuring climate resilience for coffee, changing the crop itself is paramount, and this may have to include using alternative coffee crop species. In this study we use a multidisciplinary approach to elucidate the identity, distribution, and attributes, of two minor coffee crop species from East Africa: *Coffea racemosa* and *C. zanguebariae*. Using DNA sequencing and morphology, we elucidate their phylogenetic relationships and confirm that they represent two distinct but closely related species. Climate profiling is used to understand their basic climatic requirements, which are compared to those of Arabica (*C. arabica*) and robusta (*C. canephora*) coffee. Basic agronomic data (including yield) and sensory information are provided and evaluated. *Coffea racemosa* and *C. zanguebariae* possess useful traits for coffee crop plant development, particularly heat tolerance, low precipitation requirement, high precipitation seasonality (dry season tolerance) and rapid fruit development (c. 4 months flowering to mature fruit). These attributes would be best accessed *via* breeding programs, although these species also have niche-market potential, particularly after further pre-farm selection and post-harvest optimization.

Keywords: climate change, coffee, DNA sequencing, climate profiling, coffee agronomy, plastid (chloroplast) DNA, Internal Transcribed Spacer (ITS)

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INTRODUCTION

Coffee farming and the success of the coffee value chain is constrained by numerous challenges, including fluctuating market prices, adverse weather, pests and diseases, and various social challenges. Anthropogenic climate change has a compounding influence on these factors (IPCC, 2014) and has been considered as the single overarching challenge facing long-term sustainability for coffee production (Davis et al., 2019). There are three main adaptation strategies for ensuring resilience for the coffee farming sector under climate change (Davis et al., 2021): (1) farm adaptation, by changing the air and soil microclimate on the farm, *via* various means; (2) relocation of coffee farming areas, e.g., to higher, cooler elevations; and (3) replacement, i.e., changing the

crop using new cultivars, new hybrids, or alternative species. All three strategies have merit, but relocation (2) and replacement (3) offer the most potential over the longer term, under existing climate change projections (Davis et al., 2021). Despite the substantial potential of relocation for countries with suitable elevational range, such as Ethiopia (Moat et al., 2017), or other forms of spatio-climatic intervention, there are likely to be issues around competing land-use (for communities and biodiversity) and other negative societal implications. There will no doubt be winners and losers as farms either diversify into coffee or move away from coffee, as the climate changes. The capacity for human migration is usually limited and almost certainly undesirable. At this time, changing the crop therefore represents the most suitable adaptation pathway for coffee under accelerated climate change (Davis et al., 2019, 2021). Our current coffee crop species, Arabica (*Coffea arabica*) and robusta (*C. canephora*) still provide ample coffee to supply the global value chain, but narrative from farmers across the world's coffee belt, and ongoing shortfalls in production during weather perturbations and cyclic climatic phenomena, tell of ever-increasing climate-related issues. This is a result of the specificity of the climate envelopes for Arabica (Moat et al., 2017, 2019; Davis et al., 2018, 2021) and robusta (Kath et al., 2020), the perennial nature of the crop, and the fact that coffee farming has been extended into suboptimal climatic space for these two species and their cultivars.

Amongst the 130 wild coffee species (Davis and Rakotonasolo, 2021) there could be potential amongst the underutilized species (e.g., *C. liberica*), minor crop species (e.g., *C. eugenioides*, *C. racemosa*) or the (numerous) non-utilized species, to broaden the coffee crop portfolio, and provide material for coffee crop development (Davis et al., 2019) in an era of accelerated climate change. There are 42 priority coffee species (Davis et al., 2019), worthy of further evaluation and experiment. The case of *C. stenophylla*, which has an Arabica-like taste but can grow successfully at much warmer temperatures (mean annual temperature requirement 6.2–6.8°C higher than Arabica; Davis et al., 2021), demonstrates the potential of other coffee species to prove climate resiliency crop alternatives or, perhaps more importantly, resources for breeding. Even if *C. stenophylla* coffee proves to be successful, there will still be a requirement to further broaden the climatic portfolio of coffee crop species, including those that can grow and crop under both warmer, drier and more seasonal conditions than Arabica and robusta, and even *C. stenophylla*. In this respect, the current study focused on two minor coffee crops of East Africa, viz. *C. racemosa* and *C. zanguebariae*.

Coffea racemosa was described by Loureiro (1790) based on a herbarium specimen collected in Mozambique, although this specimen has not been recovered and is now replaced by a new type (neotype) specimen from the Massingir District (Gaza Province) in southwestern Mozambique (Bridson, 2003). Given its rather unique appearance, and knowledge of its use as a crop from previous times, *C. racemosa* has been adequately covered in many classical works on coffee (e.g., Cheney, 1925; Wellman, 1961; Haarer, 1962) but is notably absent from the work of Cramer (1957) possibly because Java does not provide a favourable environment for the growth of

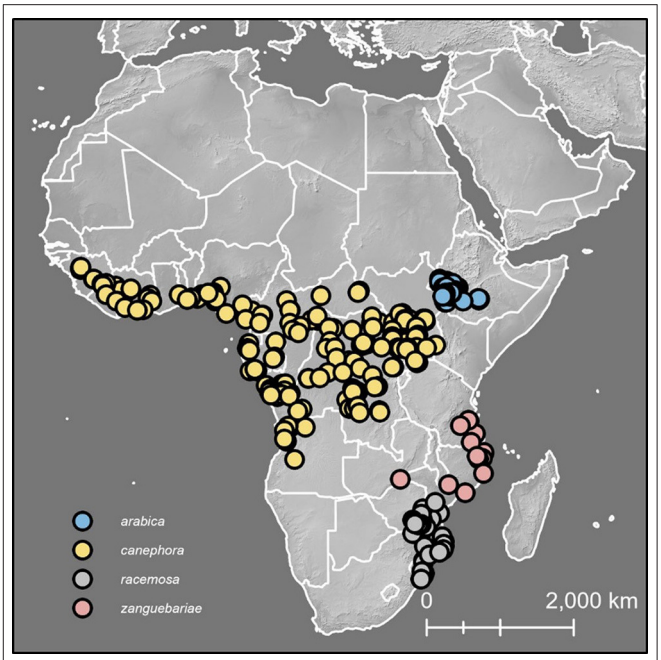

FIGURE 1 | Distribution map for *C. arabica*, *C. canephora*, *C. racemosa* and *C. zanguebariae*. Figure also indicates ground point data for climate profiling analysis (**Figures 8**, **9**).

C. racemosa. A thorough review of *C. racemosa* was provided by Guerreiro Filho (1992), covering botanical features, taxonomic classification, geography (distribution), genetics and breeding, chemistry, possible attributes for the improvement (pest and disease resistance, climate resilience, shortening of fruiting time compared to Arabica and robusta), and a brief overview of sensory qualities (flavour). Attributes of particular note reported by Guerreiro Filho (1992) were: tolerance of higher temperatures and low monthly precipitation values, mostly based on the findings of Krug (1965) and Halle and Faria (1973); hybridization success with Arabica and robusta; resistance to various coffee pests, particularly coffee leaf miner (*Perileucoptera coffeella*) and nematodes (*Meloidogyne* species) and disease; and reduction in fruiting times for interspecies hybrids. According to (Guerreiro Filho, 1992), broad scale success of *C. racemosa* as a crop species was deemed unlikely due to its very small seed (coffee bean) size, based on examination and data from Lopes (1974), which stated a mean seed size of c. 6.3 × 5.1 × 3.2 (length, width, thickness). No yield (production) figures were given by Guerreiro Filho (1992) but this is also, of course, a key factor for agronomic and value chain success.

Climate information provided for *C. racemosa* by Krug (1965) and illustrated by Guerreiro Filho (1992; **Figure 1**, areas a, b, c) reported: (a) mean monthly temperatures of 18.9–26.4°C, an annual precipitation of c. 1,600 mm, and a 4–5 month dry season (May to October), for populations occurring at 200–500 m in central Mozambique; (b) mean monthly temperatures of 21.7–28.3°C, and 1,200–1,300 mm annual precipitation for low elevation (0–200 m) coastal areas of central Mozambique;

and 19.6–27.3°C, and a 700–900 mm annual precipitation, for populations in areas in low elevation (0–200 m) coastal areas of southern Mozambique. Halle and Faria (1973) reported that between latitude 22°S and 25°S, at an elevation not exceeding 40 m, the annual precipitation for *C. racemosa* was 647 mm. Overall, these climate profiling data are useful but somewhat contradictory for a single species, particularly given that the mean annual precipitation requirement for *C. arabica* and *C. canephora* is around 1,600 m (Davis et al., 2021).

Coffea zanguebariae was also described by Loureiro (1790), based on material collected from Zanzibar, which Loureiro remarks to have been taken there by the Portuguese. Due to similarities in morphology, *C. zanguebariae* has been routinely confused with *C. racemosa* (Haarer, 1962; Guerreiro Filho, 1992), other coffee species (Cheney, 1925; Haarer, 1962), and its identity has been undoubtedly mistaken (Cramer, 1957). Chevalier (1942, 1947) was well acquainted with *C. racemosa* but included what we now know as other *Coffea* species under *C. zanguebariae*, and most notably *C. pseudozanguebariae* (Bridson, 1982); this has led to considerable confusion over the identity of this species (Laíns E Silva, 1954; Guerreiro Filho, 1992). Indeed, Guerreiro Filho (1992) clearly considered *C. racemosa* and *C. zanguebariae* to be represented by a single species. On the basis of morpho-taxonomic study, Bridson (1988, 2003) reassessed the circumscriptions of *C. zanguebariae* and *C. racemosa*, demonstrated that they were separate species, and resolved outstanding taxonomic and nomenclatural issues. Botanists working in Mozambique have generally followed the assessment of Bridson (e.g., Burrows et al., 2018). Bridson (2003) considered *C. racemosa* as indigenous to central and southern Mozambique, northern South Africa (Kwa Zulu Natal), and eastern Zimbabwe, as a plant of coastal forest, riverine forest, deciduous woodland and bushland at elevation of 0–500 m. *Coffea zanguebariae* was reported by Bridson (1988, 2003) as indigenous to southern Tanzania, northern Zimbabwe and northern Mozambique, occurring in dry deciduous forest, and riverine and coastal thicket, at an elevation of 10–350 m. Common synonyms given by Bridson (2003) for *C. racemosa* include *C. mozambiciana, C. ramosa*, and *C. swynnertonii*; and for *C. zanguebariae*, synonyms include *C. ibo*, *C. schumanniana*, and *C. zanzibarensis*. These names become important when tracing the history of these species in cultivation and commerce.

Despite the apparent taxonomic and nomenclatural clarity afforded for *C. racemosa* and *C. zanguebariae* by Bridson (1988, 2003), there remains a great deal of confusion between these species in commerce and in coffee research. The phylogenetic placement of *C. zanguebariae* remains inconclusive (Maurin et al., 2007; Davis et al., 2011); and this species has not been included amongst recent molecular sampling for the genus (e.g., Hamon et al., 2017). With respect to the possible development of *C. racemosa* and *C. zanguebariae* as either coffee crops or plant breeding resources, certainty over the circumscription of these species, and understanding the relationship between them and other *Coffea* species, is required.

In addition to the incongruences reported for climate profiles and systematic uncertainty, there is a third point of contention regarding *C. racemosa* and *C. zanguebariae*, viz. their flavour characteristics. In the review by (Guerreiro Filho, 1992) various reports are given for the flavour quality of *C. racemosa*. According to Silva (1956) *C. racemosa* provides an aromatic drink, of slightly dark colour and low caffeine; Chevalier (1947) noted from a herbarium sheet than the "Grain is considered analogous to Moka [*C. arabica*], it is better than any other;" and Carvalho (pers. comm. in Guerreiro Filho, 1992) reported an inferior quality to that of *C. arabica*. Other reports include that of Haarer (1962) who stated that: "Coffee prepared from the beans [of *C. racemosa*] is used by the local settlers and said to be of excellent quality," and Laíns E Silva (1954) that it produced an aromatic, lightly coloured beverage, with a low caffeine content, and was much favoured for batch mixing with *C. arabica*, but that this coffee was sought after in Mozambique without mixing. Published information, or otherwise, on the sensory qualities of *C. zanguebariae* appears to be non-existent.

Further points of ambiguity are apparent. Guerreiro Filho (1992; **Figure 1**) provide some basic details on the distribution of cultivated (farmed) *C. racemosa* (including *C. zanguebariae*; see above) but the distinction between cultivated and wild distributions was not made. The vernacular name "Inhambane coffee" is reported from several herbarium specimens of *C. racemosa* farmed in the region of that name in southern Mozambique (Bridson, 2003). There are early reports of the cultivation of *C. zanguebariae*: Cheney (1925) remarked that *C. ibo* (i.e., *C. zanguebariae* fide Bridson, 2003), appeared on the German southeast African market in 1893, and on Ibo island (north-eastern Mozambique) it was being grown in the 1920s as a substitute for *C. arabica*.

There remains, therefore, five main points of ambiguity for *C. racemosa* and *C. zanguebariae*, viz.: the systematic (phylogenetic) relationship between these two species, and other East Africa taxa; their climatic (and agro-climatic) requirements; their sensory qualities; their current usage as minor crops. An obvious shortfall for cultivated *C. racemosa* and *C. zanguebariae* is basic agronomic knowledge, such as harvest periods, yield, and conversion ratios to green coffee. Our objectives were, therefore, to: (1) elucidate the phylogenetic and spatio-phylogenetic relationship between *C. racemosa* and *C. zanguebariae* and to confirm or refute their existence as separate species; (2) model the climate profiles for these species, with a view to gaining a better understanding of their climatic requirements and agro-climatic suitability; (3) to provide preliminary sensory (flavour) information; and (4) to provide basic agronomic data. To undertake these objectives, we utilized plastid and ITS sequence data, climate profiling analyses using ground-point data, field and herbarium observation (of wild and farmed plants), farm surveys, literature review, and preliminary sensory information on beverage quality using standard industry procedures and narratives from coffee tasting professionals.

METHODS

Fieldwork and Other Observation Data

Farm visits were made between 2016 and 2020, in northern South Africa (Kwa-Zulu Natal), at Hluhluwe (for *C. racemosa*) and in north-eastern Mozambique (Cabo Delgado Province),

on Ibo Island and Quirimba Island and near Pemba on the mainland (for *C. zanguebariae*). Study of wild populations was undertaken north of Durban in South Africa (*C. racemosa*) and west of Pemba in north-eastern Mozambique (*C. zanguebariae*). Location, morphology, ecology, and agronomy data were also collected *in situ*. Herbarium specimens were consulted from five herbaria [BM, BR, K, P, WAG; codes follow Holmgren et al. (1990) and Thiers (2019)] for additional information, including habitat, vegetation, environment, morphology, cultivation history and trade.

Data for Mapping and Climate Profiling

Ground points derived from herbarium specimens and field survey were used to provide the data for the climate profiling analyses and to produce distribution maps for indigenous locations of *C. arabica* and *C. canephora*, and indigenous and cultivated (farmed) locations of *C. racemosa* and *C. zanguebariae*. Herbarium data are well suited for providing these data because they are verifiable in space (location), time (date) and form (species identity). We consulted herbarium specimen records from five herbaria (BM, BR, K, P, WAG). Location data from the herbarium specimens were georeferenced (if not already available), manually checked for geolocation accuracy (1 km or less) using GoogleEath® and corrected if necessary. For the distribution maps and climate profiling analysis we used a dataset of 1,087 ground point records, comprising 711 records for *C. arabica*, 304 for *C. canephora*, 50 for *C. racemosa*, and 22 for *C. zanguebariae*. We used indigenous and cultivated datapoints for *C. racemosa* and *C. zanguebariae* as they were either overlapped or contiguous. Disaggregated in this way, the number of datapoints for each class was 36 wild and 14 cultivated for *C. racemosa*, and 15 wild and seven cultivated for *C. zanguebariae*.

Distribution maps were produced in ArcGIS Pro 2.6.1 [Environmental Systems Research Institute (Esri), 2020], using background and country data from Natural Earth (https://www.naturalearthdata.com/).

Climate Profiling Analyses

For these analyses we resampled all specimen data to remove duplicates within 1 km of each other, reducing the total number of records used from 1,087 to 463 (193 for *C. arabica*, 200 for *C. canephora*, 50 for *C. racemosa*, and 20 for *C. zanguebariae*. To understand the fundamental climatic requirements, the statistics package R (R Core Team, 2016) was used to sample specimen data against 19 Bioclim variables (Busby, 1991) from the CHELSA dataset (Karger et al., 2017). For an overview of climatic parameters, we selected the following three Bioclims: Bio1 Annual mean temperature, Bio12 Annual Precipitation, and Bio15 Precipitation Seasonality. Scatter and density plots were plotted using R (R Core Team, 2016), using the ggplot2 (Wickham, 2016) and ggpubr packages (Kassambara, 2020). For validation purposes, our modelled annual temperatures (from Bio 1), annual precipitation (Bio12) and precipitation seasonality (Bio15) used to produce **Figures 1**, **2** were compared against publicly available monthly mean temperature precipitation charts for East Africa and published data (Laíns E Silva, 1954; Alègre, 1959; DaMatta, 2004; DaMatta and Ramalho, 2006; Kath et al., 2020). Our modelling methods have been shown to provide climate metrics that are similar to those provided for coffee species in cultivation, produced by direct measurement and other means (Davis et al., 2021).

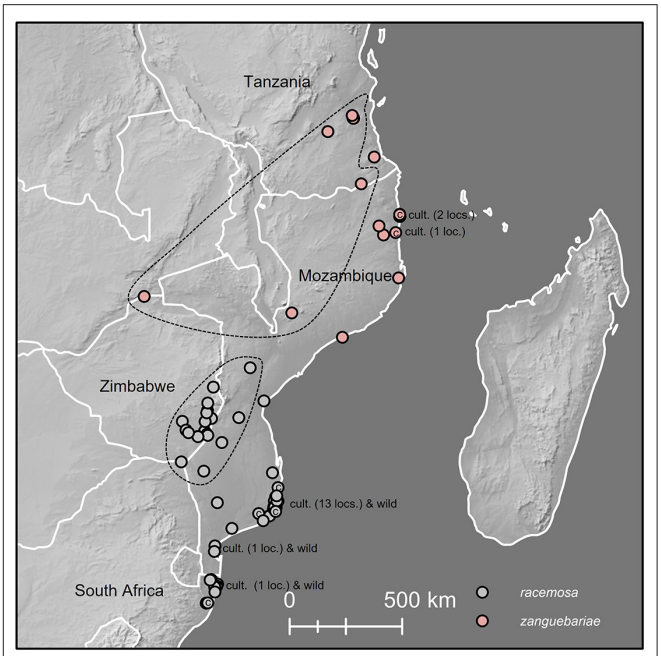

FIGURE 2 | Distribution map for *C. racemosa* and *C. zanguebariae*. Dotted lines indicate localities above 150 m for *C. racemosa* and 200 m for *C. zanguebariae*. Cult. (cultivated) indicates localities of farmed coffee; loc./locs. = locality/localities.

Bioclim variables (Busby, 1991) from the CHELSA dataset provides modelled climate data at a grid size of 1 km, but these data are modelled and gridded based on local weather stations, which although numerous in some countries are usually spaced many kilometres apart, and are mostly located in urban areas. Depending on local topological features (outcrops, hills, valleys, slope, and aspect), other features of the landscape (rivers, height of the water table, soils, rock outcrops) the local climate and microclimate may differ considerably. Other data sources were therefore used to understand local and microclimate influences (see Fieldwork and Other Observation Data, above).

DNA Sampling, Extraction, Sequencing, and Data Analysis

To assess molecular variation between *C. zanguebariae* and *C. racemosa*, we analysed the plastid regions *trn*L-*trn*F (*trn*L intron and *trn*L-*trn*F intergenic spacer), *rpl*16 intron and *acc*D-*psa*I intergenic spacer, and the nuclear Internal Transcribed Spacer (ITS). These markers have the ability to distinguish between African *Coffea* species, and identify recently formed hybrids *via* differential inheritance of plastid and nuclear genomes (Maurin et al., 2007; Davis et al., 2020). In addition to the accessions already available in GenBank for the two species,

TABLE 1 | List of DNA sampling and accession data.

Name-Code	Locality	Reference/Voucher	trnL-trnF	rpl16	accD-psaI	ITS
C. costatifructa	Tanzania	Maurin et al., 2007	DQ153840	DQ15372	DQ153473	DQ153604
C. sessiliflora	Tanzania	Maurin et al., 2007	DQ153818	DQ153700	DQ153451	DQ153579
***C. racemosa* (1) cult.**	Brazil cult.	Davis et al., 2020	MN715169	MN715224	MN715197	MN719961
***C. racemosa* (2)**	Mozambique	Davis et al., 2020	DQ153863	DQ153745	DQ153496	DQ153627
***C. racemosa* (3)**	Mozambique	Davis et al., 2020	DQ153831	DQ153713	DQ153464	DQ153595
***C. racemosa* (4)**	Mozambique	Davis et al., 2020	DQ153864	DQ153746	DQ153497	DQ153628
***C. zanguebariae* Moz 1 (Z4)**	Mozambique,	NE Mozambique, near Metoro, Groenendijk 884 (K)	**MZ675750**	**MZ675745**	**MZ675754**	**MZ619086**
***C. zanguebariae* (Moz 2) cult. (Z5)**	Mozambique,	Ibo Island (farm), seed (K)	N.A.	**MZ675746**	**MZ675755**	**MZ619087**
***C. zanguebariae* (Moz 3) cult. (Z6)**	Mozambique	Ibo Island, Ibo (garden), seed (K)	**MZ675751**	**MZ675747**	**MZ675756**	**MZ619088**
***C. zanguebariae* T1 (Z1)**	Mozambique	Taratibu, near Metoro (K)	**MZ675752**	**MZ675748**	**MZ675757**	**MZ619089**
***C. zanguebariae* T2 (Z2)**	Mozambique	Davis et al., 2020	MN715171	MN715226	MN715199	MN719963
***C. zanguebariae* T3 (Z3)**	Mozambique	NE Mozambique (K)	**MZ675753**	**MZ675749**	**MZ675758**	**MZ619090**

Accession numbers in bold indicate material sequenced in this study.

one for *C. zanguebariae* and four for *C. racemosa* (Davis et al., 2020), we analysed five further samples for *C. zanguebariae* (see **Table 1**). The *C. zanguebariae* accession used by Maurin et al. (2007) and Davis et al. (2011) was omitted as its phylogenetic position is ambiguous, most likely due to partial sequences for *trnL–F* intron, *trnL–F* intergenic spacer (IGS), and *accD–psaI* IGS. A final dataset of four accessions for *C. racemosa* and six accessions for *C. zanguebariae* was used for illustrative purposes, with *C. costatifructa* and *C. sessiliflora* used as outgroups (**Table 1**). Previous analyses of global and African datasets shows that accessions of *C. racemosa* (Maurin et al., 2007; Davis et al., 2011; Hamon et al., 2017) and one accession of *C. zanguebariae* (Davis et al., 2020) belong to a clade of low elevation East African Coffea species, comprising *C. costatifructa*, *C. pocsii*, and *C. sessiliflora*.

We extracted DNAs from dried leaves with a modified CTAB protocol (Doyle and Doyle, 1987) and purified them using a QIAquick purification kit (QIAGEN). DNA quality was checked on an agarose gel by electrophoresis. Polymerase chain reactions were conducted as described in Maurin et al. (2007); Sanger sequencing and alignments were carried out following Davis et al. (2020). To reconstruct phylogenetic relationships among the accessions of *C. zanguebariae* and *C. racemosa*, the alignments obtained for the plastid and the nuclear regions were concatenated and analysed using Bayesian inference, as implemented in the software MrBayes v.3.2.7 (Huelsenbeck and Ronquist, 2001) with the Beagle library (Ayres et al., 2012). The analysis was carried out using the CIPRES Science Gateway ver. 3.3 (Miller et al., 2010). In MrBayes, we assumed a GTR model of molecular evolution with gamma rate of variation and the sampling parameters were set as following: two runs and four chains for 20,000,000 generations with a relative burn-in of 25%, sampling the chains every 1,000 generations. A maximum clade credibility tree was constructed using FigTree ver. 1.4.4 (Rambaut, 2018).

Flavour Assessment

Several samples of *C. racemosa* from Hluhluwe (South Africa) and two bulk samples of *C. zanguebariae* from Ibo Island (Mozambique) were obtained for flavour (aroma and taste) assessment purposes. The *C. racemosa* samples were supplied by Cultivar Coffee (Hluhluwe Kwa-Zulu Natal, South Africa) a farm specialising in the cultivation of this species; the samples of *C. zanguebariae* were purchased directly from Ibo Island (northern Mozambique). The *C. racemosa* samples were wet processed (washed coffee) and carefully processed to optimize quality. The *C. zanguebariae* samples were sundried locally (on Ibo Island) but were unlikely to have received optimal processing, considering the coffee was not for export but rather for local use.

The samples were sent to two independent coffee professionals and two commercial sensory laboratories, one belonging to a coffee supplier and the other to a roaster and retailer (see Acknowledgements for details). Given that no protocols exist for cupping the two study species, which are inherently very different physically (and possibly chemically) from Arabica and robusta coffee, we asked that the roasting be optimized as best as possible. Simplified or modified versions of the Speciality Coffee Association (SCA) protocol https://sca.coffee/research/protocols-best-practices) were used, with sensory terminology broadly following, where possible, the SCA Coffee Taster's Flavour Wheel (Spencer et al., 2016). No scoring was applied to evaluate the coffees. The aim was not to provide a detailed sensory assessment of each sample and species, but rather to attain a general overview of flavour quality, and cup profile (including flavour notes).

RESULTS

Geographical Distribution and Habitat of *C. racemosa* and *C. zanguebariae*

A distribution map for naturally occurring (wild) *C. arabica, C. canephora*, and wild and cultivated (farmed) *C. racemosa* and *C. zanguebariae* is shown in **Figure 1**; a higher resolution distribution map for *C. racemosa* and *C. zanguebariae* is given in **Figure 2**. The natural distributions of these four species do not overlap.

The indigenous geographical distribution of *C. racemosa* is central and southern Mozambique, eastern Zimbabwe and northern South Africa (Kwa-Zulu Natal), as reported by Bridson (2003), between latitude 18°20′-28°30′ (**Figure 2**). Two distinct ecological niches were identified *via* mapping (including visualization using satellite imagery (GoogleEarth®), herbarium data, and climate profiling (see below): (1) low elevation (<200 m), seasonal (deciduous) coastal forest and bushland, often associated with sandy soils; and (2) low to medium elevation [200–600(−780 m)], seasonal deciduous and semi-evergreen forest, in close proximity to rivers.

Coffea zanguebariae is indigenous in southern Tanzania, northern Zimbabwe and northern Mozambique, between latitude 8°20′-17°30′ (**Figure 2**). Like *C. racemosa*, two distinct ecological niches were identified *via* mapping (including visualization using satellite imagery (GoogleEarth®), herbarium data, and personal observation (see above): (1) low elevation (5–100 m), seasonal (deciduous) coastal forest and bushland, often associated with sandy soils; and (2) low to medium elevation [100–380(−680 m)], seasonal deciduous and semi-evergreen forest, either in close proximity to rivers or amongst rocks or close to rock outcrops. Unlike *C. racemosa*, the climate profiling analysis (see below) did not identify a distinct bimodality, but this may be due to lower number of ground points, compared to *C. racemosa*. Herbarium collections made close to the town of Metoro in northern Mozambique (at c. 350 m) did not state habitat information, but direct observation shows that *C. zanguebariae* grows at the base of large rock outcrops (inselbergs), and near ephemeral water sources in places that are cooler, more humid (and with improved water availability) compared to nearby drier (Miombo) woodland that characterizes the vegetation of the area.

Morphological Review

A summary of the key morphological features of *C. racemosa* and *C. zanguebariae*, based on literature (Bridson, 1988, 2003; Burrows et al., 2018), herbarium survey, and fieldwork, is given in **Table 2**. Some morphological details are illustrated in **Figures 3–6**. Overall, *C. zanguebariae* is a larger and more robust plant compared to *C. racemosa*, being taller (and with a larger stem diameter), with larger leaves, more fruits per inflorescence (and correspondingly per axil and node), and larger seeds. Farmed material of *C. racemosa* shows distinct variation in seed size and shape (**Figures 4B,C**). Farmed *C. zanguebariae* in northern Mozambique shows considerable variation in fruit and seed size (**Figures 6A–C**); seed size dimorphism in wild *C. zanguebariae* was reported by Bridson (2003). Both species are deciduous, but may retain their leaves through the dry season in some locations, and in seasons when the dry season is not severe. In farmed conditions, some plants of *C. racemosa* may lose their leaves, whereas neighbouring plants may not (C. Denison pers. observ.).

DNA Analysis

We obtained molecular data for all the plastid and nuclear regions investigated, except the *trn*L-*trn*F in one *C. zanguebariae* sample (M2). Our concatenated plastid matrix included 3,100 characters, and our ITS matrix 792 characters (12 accessions in total). The maximum clade credibility tree resulting from the Bayesian analysis had posterior probabilities (PP) > 0.9 for all clades (**Figure 7**). A sister relationship for *C. racemosa* and *C. zanguebariae* is strongly supported (PP = 1), as are the multiple species-specific samples for each species (PP = 1).

Climate Profiling

Our modelled climate data analysis for wild and cultivated *C. racemosa* gives a mean annual average temperature of 22.9°C and a mean annual precipitation of 807 mm; and for *C. zanguebariae* 24.8°C and 998 mm. For the wild occurrences of the two main coffee crop species, *C. arabica* has a mean annual average temperature of 18.7°C and a mean annual precipitation of 1,614 mm, and for *C. canephora* 23.7°C and 1,596 mm (**Table 3**). The distribution and density of data points for these two variables, for the four species, are shown in **Figures 8**, **9**. Disaggregated data for cultivated vs. wild for *C. racemosa* and *C. zanguebariae* show slightly warmer mean annual temperatures (Bio 1) for cultivated datapoints (0.7 and 1.1°C, respectively), wetter conditions (mean annual precipitation, Bio 12) for cultivated *C. racemosa* (916 vs. 764 mm) and marginally drier conditions for cultivated *C. zanguebariae* (969 vs. 990 mm). Rainfall seasonality (Bio 15) is highest (more seasonal) in *C. racemosa* and *C. zanguebariae* compared to *C. arabica* and *C. canephora*, and there is a second major peak for rainfall seasonality (Bio 15) in *C. racemosa*, with a mean value midway between *C. arabica* and *C. canephora* (**Table 3**; **Figure 9**). The lower valued precipitation seasonality (Bio 15) peak for *C. racemosa* (**Figure 4**) is due to the lower value returned by coastal datapoints (mean value of 55); the inland points (**Figure 2**) having a higher precipitation seasonality (mean value 74). Spot checks against publicly available monthly mean precipitation charts for Mozambique and published data (Laíns E Silva, 1954) confirm the distinct precipitation seasonality (i.e., a distinct wet and distinct dry season) for *C. racemosa* and *C. zanguebariae*.

Farming of *C. racemosa* and *C. zanguebariae*

Coffea racemosa is currently being farmed at small scale at Kwa Zulu Natal in South Africa (Hluhluwe) and probably on isolated farms in southern Mozambique; *C. zanguebariae* is being cultivated on Ibo and Quirimbas Islands, and mainland northern Mozambique (near Pemba). Cultivation of *C. zanguebariae* on Ibo and Quirimbas Islands (as Ibo coffee) is long-established and probably dates back to at least the 1890s (Cheney, 1925), as reported in the Introduction. Cheney (1925) also mentions, based on an earlier reference (Lanessan, 1886), that in 1880 9,300 kg of "excellent coffee seeds" from *C. zanguebariae* were harvested on Nossibe (Nosy Bé) an Island off the north-western coast of Madagascar. On reviewing Lanessan (1886), this statement seems to be erroneous, as on page 44 Lanessan states: "In the wild, *Coffea zanguebariae* Lour. is found on the island, whose grain has a very delicate flavour. Cultivation trials have been made with various varieties of Bourbon coffee (*C. arabica*) trees, which grow vigorously, even without shelter, and give a high estimated gain in the country. In 1880, 9,300 kg were

TABLE 2 | Distinguishing morphological characteristics, and distribution and ecology summary for *C. racemosa* and *C. zanguebariae*.

Character	*Coffea racemosa*	*Coffea zanguebariae*
Habit	Treelet (shrub-like), 1–3.5(−5) mm tall	Treelet to small tree, 3–6 m tall
Young stems	Puberulent (lightly covered with fine hairs)	Glabrous (no hairs)
Leaves (habit)	Deciduous, or semi-deciduous	Deciduous, or semi-deciduous, or semi-persistent
Leaves (petioles)	1–2.5 mm long	3–6.4 mm long
Leaves (margins)	Often undulate (wavy)	Flat or slightly wavy (undulate)
Leaves (length × width)	2.2–7 × 1–3.4 cm	5.4–14 × 2.2–7.9 cm
Leaves (shape)	Narrowly elliptic to broadly elliptic	Broadly elliptic, or elliptic
Leaves (secondary veins)	4–6	5–6(−7)
Flower (part numbers)	5–9(−12)	6–8
Flower (corolla)	Light pink, turning white	Light pink, turning white, or white
Inflorescence	Inflorescences 1–2 per axil, each with 1–3 flowers	Inflorescences 1 per axil, each with 2–5 flowers
Fruit (number)	1–3 fruits per axil (2–6 per node)	2–5 fruits per axil (4–10 per node)
Fruit size dried/fresh	0.9–1.6 × 0.6–1.2	0.9–2.2 × 1.3–1.7 cm
Fruit (colour)	Slightly to moderately ribbed in the green and red stage, smooth and purplish black when ripe (mature)	Markedly ribbed in the green and red stage, smooth or lightly ribbed and purplish black when ripe (mature)
Seeds	3.9–7 × 3.2–5.5	6–10 × 5–6 cm
Distribution	Central and southern Mozambique, eastern Zimbabwe and northern South Africa (Kwa-Zulu Natal),	Southern Tanzania, northern Zimbabwe and northern Mozambique.
Ecology	Low elevation (0–200 m), seasonal (deciduous) coastal forest and bushland, often associated with sandy soils; and (2) low to medium elevation [200–600(−780 m)], seasonal deciduous and semi-evergreen forest, in close proximity to rivers.	Low elevation (5–100 m), seasonal (deciduous) coastal forest and bushland, often associated with sandy soils; and (2) low to medium elevation [100–380(−680 m)], seasonal deciduous and semi-evergreen forest, either in close proximity to rivers or amongst rocks or close to rock outcrops

Figures in parentheses directly after measurement indicate extreme values.

FIGURE 3 | *Coffea racemosa*. **(A)** Habit of cultivated plant, with immature fruits; **(B)** Flowers, mature, 5-lobed variant; **(C)** Fruits, approaching full maturity. Images: Charles Denison.

FIGURE 4 | *Coffea racemosa*. **(A)** Fresh, fully mature (ripe) fruits; **(B)** seeds, showing variation in size; **(C)** seeds (left to right) of *Coffea racemosa*, compared to *C. canephora* (robusta) and *C. arabica* (Arabica). Images: **(A)** Charles Denison); **(B,C)** Aaron Davis.

collected." And on page 833, Lanessan (1886) states that *Coffea zanguebariae* grows wild on Nosy Bé'. Clearly then, the assertion made by Cheney (1925) is incorrect, as *C. zanguebariae* does not grow wild on Nosy Bé, where only a single endemic, wild species is found, viz. *C. pervilleana* (Davis et al., 2006) and, moreover, Lanessan (1886) is referring to the yield of *C. arabica* (cv. "Bourbon") on Nosy Bé, not *C. zanguebariae*.

FIGURE 5 | *Coffea zanguebariae.* **(A)** Shoots of a cultivated plant, with immature fruits and ripening fruits (red), cultivated near Pema, north-eastern Mozambique; **(B)** Leaves of a wild plants, for size comparison; **(C)** Habit of wild plant. **(B,C)** images taken at Taratibu, near Metoro, near Pemba, north-eastern Mozambique (images: Aaron Davis).

TABLE 3 | Climate profiling summary with mean values and disaggregated mean values for wild and cultivated (*C. racemosa* and *C. zanguebariae*), and coastal (low elevation) and inland (higher elevation) for *C. racemosa*.

Species	Mean temp. (°C)	Mean precipitation (mm)	Precipitation seasonality (CHELSA)
C. arabica	18.7	1,614	58
C. canephora	23.7	1,596	56
C. racemosa	22.9	807	69
C. zanguebariae	24.8	998	90
Disaggregated (wild vs. cult.)			
C. racemosa—wild	22.9	764	74
C. racemosa—cult.	23.6	916	55
C. zanguebariae—wild	24.8	990	90
C. zanguebariae—cult.	25.9	969	98
Disaggregated (coastal vs. inland)			
C. racemosa—coastal	23.3	915	56
C. racemosa—inland	22.8	631	90

See Methods for further details.

FIGURE 6 | *Coffea zanguebariae.* **(A)** Shoots of a cultivated plant, with immature fruits—left, showing upper (adaxial) surface of leaves; right, showing lower (abaxial) surface of leaves; **(B)** Ripening and almost ripe (mature) fruits, from farmed plants; **(C)** Fruits, and seeds enclosed in endocarp (parchment)—immediate left, immature fruits (green), uppermost, dried fruits (cherry) and lowermost, parchment coffee, indicating variation in parchment/seed size; **(D)** unroasted (above) and roasted (below) seeds of *C. zanguebariae* (four seeds to left), and compared to those of *C. racemosa* (right). Images **(A–C)** taken in north-eastern Mozambique, **(A,C)** on Ibo Island and **(B)** near Pemba, and **(D)** in the UK. Images: Aaron Davis.

We were unable to verify whether cultivation of *C. zanguebariae* (as Zanzibar coffee) still occurs on Zanzibar (Loureiro, 1790; Cheney, 1925), or whether it was brought there from Ibo island, or vice versa, by boat, by Portuguese and other traders, or originated from mainland Tanzania. Given that both Ibo and Zanzibar were key trading ports, a dissemination of cultivated material from island to island is more likely. If we accept the opinion of Loureiro (1790), that *C. zanguebariae* was taken to Zanzibar by the Portuguese, this species has been cultivated there prior to 1790. The earliest record for the cultivation of *C. racemosa* is uncertain, although its use as coffee was noted by Hiern (1876) and it is clear from herbarium records (from 1937) that this species was quite widely cultivated in the Inhambane Province of Mozambique (as Inhambane coffee) in the first half of the twentieth century. We have not been able to verify whether cultivation of *C. racemosa* persists in and around Inhambane, although personal communication with various persons suggest that it is being farmed there on a local scale.

Basic Agronomic Information

The following observation and data records for *C. racemosa* were made at Hluhluwe, Kwa-Zulu Natal, South Africa, which has a mean annual precipitation of c. 530 mm (with 3 months precipitation of <50 mm, and 6 months <100 mm), and a mean annual temperature of 22.2°C (December, January and February are the hottest months, with mean maximum temperatures of c. 28/29°C). The tree spacing for farmed plots is 1.5 × 2.5 m or 1.5 ×1.5 m. Flowering occurs over a short period from mid-August to mid-September, with flower initiation dependent on the first rains, with fruit development following shortly afterwards. Each flower only lasts about 3 days, and all flowering is over in about 3 weeks. By early November the fruit is at full size, after a rapid ripening and harvest in late November to the end of December of the same year, although small volumes of fruit are harvested in early to mid-January. The period from flowering (anthesis) to harvest (fruit ripening), for farmed plants of this species is thus around 4 months; the same observations are made for wild plants of *C. racemosa* (Bridson, 2003). At around 7 years old, each (i.e., not fully mature) of the trees under observation produced a mean value of 111 g of ripe fruit (fresh cherry), which converts to c. 12 g of clean, green coffee, giving a conversion rate of 9.4:1 (the farm works on a 10:1

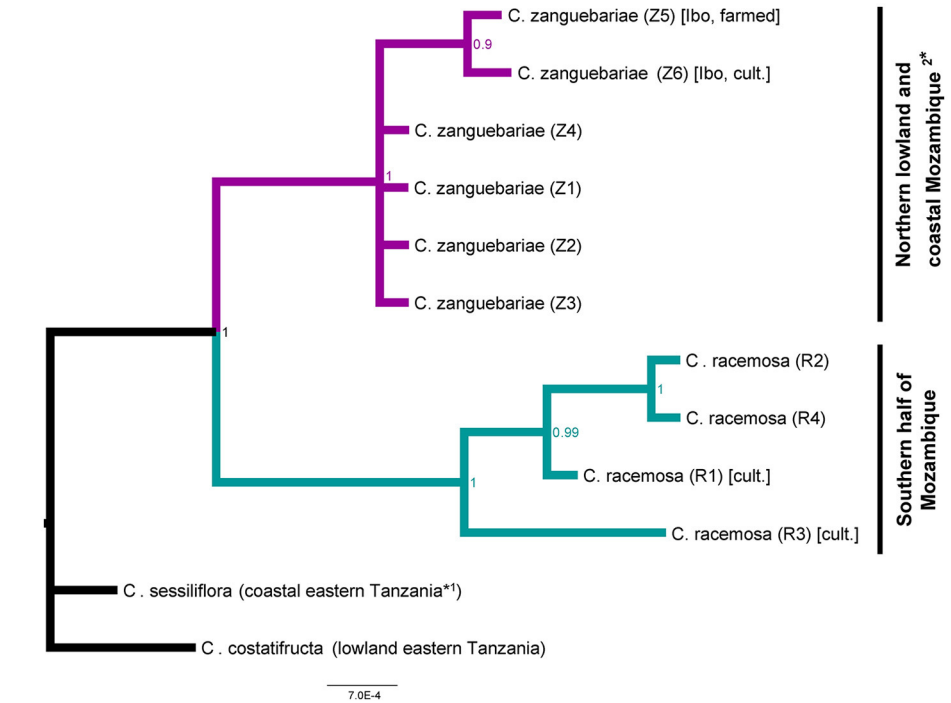

FIGURE 7 | Combined ITS and plastid maximum clade credibility tree. Bayesian posterior probabilities are indicated at nodes. See **Table 1** for accession information. Notes: 1* *Coffea sessiliflora* also occurs in coastal eastern Kenya; 2* *C. zanguebariae* also occurs in southern Tanzania, and northern Zimbabwe.

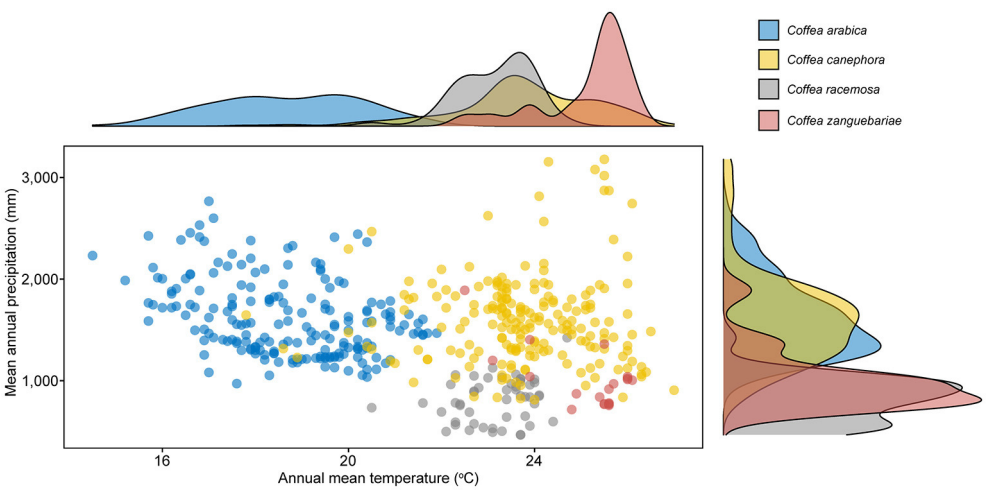

FIGURE 8 | Scatter and density plots of modelled mean annual temperature (CHELSA bio10_01) vs. total mean annual precipitation (CHELSA bio10_012). Mean values in parentheses: Arabica (*C. arabica*; 18.7°C/1,614 mm); robusta (*C. canephora*; 23.7°C/1,596 mm); wild and cultivated *C. racemosa* (22.9°C/807 mm) and wild and cultivated *C. zanguebariae* (24.8°C/998 mm). Disaggregated values for wild and cultivated (*C. racemosa* and *C. zanguebariae*), and coastal (low elevation) and inland (higher elevation) for *C. racemosa* are given in **Table 3**. See Methods for further details.

conversion), when using the wet processing method. The mean loss from fresh cherry to pulped coffee (i.e., with the fleshy part removed) is 59.1%; the mean loss from pulped coffee to parchment is 63.9%; and the mean loss from parchment to green is 28.2%.

The following observation and data records for *C. zanguebariae* were made on Ibo Island, northern Mozambique, which as mean annual precipitation of c. 950 mm (with 7 months precipitation of <50 mm, and a mean annual temperature of 26°C (December, January and February are the

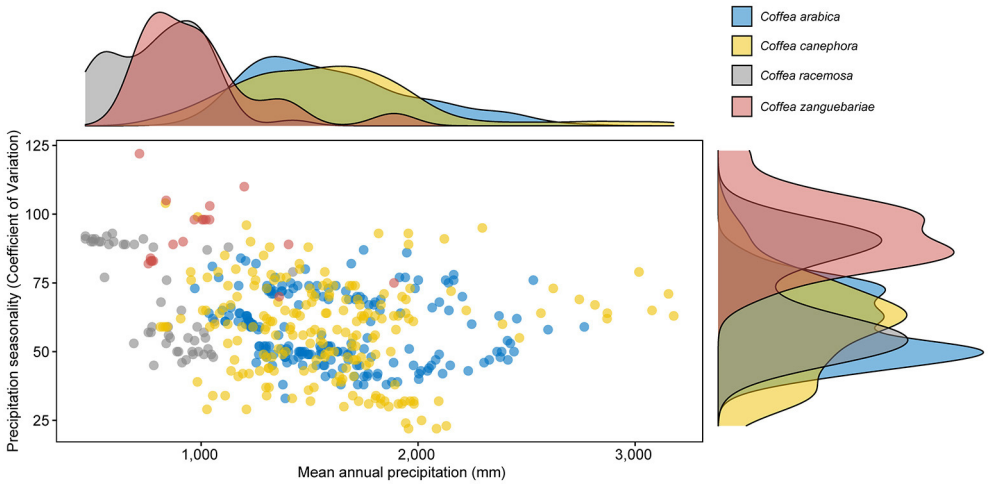

FIGURE 9 | Scatter and density plots of modelled total mean annual precipitation (CHELSA bio10_012) vs. precipitation seasonality (CHELSA bio10_015). Mean values in parentheses: Arabica (*C. arabica*; 1,614 mm/58); robusta (*C. canephora*; 1,596 mm); wild and cultivated *C. racemosa* (807 mm/69) and wild and cultivated *C. zanguebariae* (998 mm/90). Disaggregated values for wild and cultivated (*C. racemosa* and *C. zanguebariae*), and coastal (low elevation) and inland (higher elevation) for *C. racemosa* are given in **Table 3**. See Methods for further details.

hottest months, with mean maximum temperatures of c. 32°C). The tree spacing for farmed plots is 2.5–3 × 2.5–3 m. Flowering occurs over a short period from December to January, with flower initiation dependent on the first major rains. The fruits are almost fully sized by February and March, and harvesting occurs from March to April. On the neighbouring Quirimba island flowering and fruiting times may be 2–4 weeks earlier. The time period from flowering to harvest is around 4 months, as observed for wild plants of *C. zanguebariae* (Bridson, 1988). Mature trees produce 3–4 kg of fresh cherry, which converts to 600–800 g of clean, green coffee at a conversion rate of 5:1, or 300–400 g (at a 10:1 conversion). Mean losses from fresh to dried cherry, and from dried cherry to clean coffee were not recorded.

Herbarium survey data reveals that *C. zanguebariae* was probably cultivated in small quantities in central Mozambique at Inhambane, although the specimens for this are incomplete and so their identification is provisional (Bridson, 1988, 2003). We could not find any specimens to support historical reports (Cheney, 1925) of *C. zanguebariae* being cultivated on Zanzibar (Tanzania) and Ibo Islands (Mozambique), although we have no reason to doubt this. Observation on (and testimony from) Ibo Island, provides evidence of long-established cultivation of *C. zanguebriae*. Numerous collections from 1937 show that *C. racemosa* was cultivated on several farms close to and within an 80 km radius of Inhambane. One specimen states that the plants being grown were 35 years old, inferring that *C. racemosa* had been in cultivation in that region since at least the beginning of the twentieth century. These data generally confirm the historical cultivation information provided by Guerreiro Filho (1992) for these species.

Flavour

All four tasting panels agreed that the *C. zanguebariae* samples had undergone sub-optimal harvesting, processing, and storage, as evidenced by physical and flavour defects. One sample was notably aged, possibly from the previous seasons harvest. Nevertheless, it was still possible to assess these two samples and make valued judgements on coffee flavour. All four panels remarked that better processed samples of *C. zanguebariae* would be of interest, and two panels noted that further work on the harvesting and processing of *C. racemosa* would be worthwhile. All four panels experienced difficulties with roasting due the small size of the beans (seeds). Ideally, a range of roasting levels and profiles would have been preferred, as this has been shown to gain a better understanding of optimal beverage quality in minor coffee crop species and wild coffee species (Davis et al., 2021; B. Bertrand and D. Mieulet pers. comm.). Combined sensory results from the four panels are as follows, but with some of the extremely negative notes (which are assumed to be the result of sub-optimal harvesting, processing, and storage) removed for the two samples of *C. zanguebariae*.

Coffea racemosa: dry aroma (freshly ground coffee): spice, sweet herbs, liquorice; body: light to medium; acidity: light; flavour notes: blackcurrant, spiced wine, spice, cannabis, star anise, liquorice, buchu (see below), sweet-cake-like, herb-like, cinnamon, cloves, camphor, violet florals, cereal, mint. Buchu, little-known outside South Africa, are plants of the genus *Barosma* (Rutaceae) and especially *Barosma betulina*; the leaves and exracted oils are noted for their smell and tasting notes of blackcurrant, spice, and a mixture of rosemary and peppermint.

Coffea zanguebariae: dry aroma: herbal, light eucalyptus, liquorice. Body: light to medium; acidity: light; flavour notes: herbal, savoury, lavender, jasmine, aniseed, liquorice, dark chocolate, spice (cardamon), eucalyptus, medicinal, vanilla, mint. Several cups of coffee were consumed on Ibo Island (A. Davis pers. observ.), including filter, cafetiere and espresso: the quality and flavour was highly variable,

but included cups showing considerable promise and remarkable uniqueness.

We found a single example of a flavour appraisal of *C. racemosa* by a coffee roaster/retailer (https://www.coffeereview.com/review/south-africa-elephant-coast-estate-coffea-racemosa/). On the basis of a blind tasting, following a medium-light roast of coffee purchased from South Africa (Hluhluwe), their assessment notes are as follows (with some minor editing): richly bittersweet, deep-toned; flavour notes: hop flowers, pink peppercorn, tangerine zest, quince, fresh-cut fir in aroma and cup; sweet-savoury structure with friendly, accessible acidity; satiny-smooth mouthfeel; the bittersweet finish centres around tangerine zest and quince; basically sweet, this coffee's main impulses are a unique and intriguing citrus-zest-like bittersweetness with savoury floral underpinnings.

DISCUSSION

Species Status and Systematic Affinities

Our DNA analyses infer that *C. racemosa* and *C. zanguebariae* are two closely related but separate entities, which given their morphological (**Table 1**) and molecular (**Figure 7**) dissimilarity should be recognized at the level of species, supporting the taxonomic conclusions of Bridson (1988, 2003). Based on our work and previous molecular analyses (Maurin et al., 2007; Davis et al., 2011, 2020; Hamon et al., 2017) we know that these two species belong to a well-supported clade of low to mid elevation East African *Coffea* species (Davis et al., 2006), comprising *C. costatifructa* (eastern Tanzania; 10–700 m), *C. pocsii* (eastern Tanzania; 270–600 m), *C. racemosa* (eastern Zimbabwe, Mozambique, South Africa (Kwa Zulu Natal; 0–600 m), *C. sessiliflora* (south-eastern Kenya and eastern Tanzania; 10–500 m) and *C. zanguebariae* [southeaster Tanzania, north-eastern Mozambique; 10–350(−600) m].

Based on taxonomic literature review (Bridson, 1988, 2003), herbarium specimen observation and field work for *C. racemosa* and *C. zanguebariae*, it is clear that species can be easily distinguished morphologically (**Table 1**). Overall, *C. zanguebariae* is a larger and more robust plant compared to *C. racemosa*, being taller (and with a larger stem diameter), with larger leaves, more fruits per inflorescence (and correspondingly for each axil and node), and larger fruits and seeds. The shoots of *C. zanguebariae* lack the fine covering of short hairs evident on the shoots of *C. racemosa*, which is a useful criterion for differentiating between sterile plants (i.e., flowers or fruits absent), and even sterile plants lacking leaves, (i.e., during the dry season).

With the species relationships for these species resolved, and with additional knowledge from literature review and field observation, it is possible to confidently assign common (vernacular) names to these species. The vernacular names for *C. racemosa* include: Inhambane coffee; and for *C. zanguebariae*: Ibo coffee and Zanzibar coffee.

Climate Profiling

The climate profiling analysis returned a mean annual average temperature and a mean annual precipitation of 22.9°C/807 mm for *C. racemosa* (wild and cultivated), and 24.8°C/ 998 mm *C. zanguebariae* (wild and cultivated). Disaggregated values for wild and cultivated occurrences are given in **Table 3**. For the indigenous occurrences of the two main coffee crop species, *C. arabica* returned values of 18.7°C and 1,614 mm, and for *C. canephora* 23.7°C and 1,596 mm (**Table 3**; Davis et al., 2021). These data support assumptions and reports of tolerance to high temperatures and low precipitation (Laíns E Silva, 1954; Krug, 1965; Halle and Faria, 1973; Guerreiro Filho, 1992), which would, of course, be expected given the natural distribution of these two species. The higher precipitation values accorded to these species by Krug (1965) and as also reported in Guerreiro Filho (1992), i.e., of 1,300 mm and 1,600 mm, are considered erroneous; these amounts being similar to those experienced by indigenous and cultivated *C. arabica* and *C. canephora*, which occur natutrally in much wetter areas of Africa.

Other climate parameters are of note, including seasonality, and mean absolute maximum temperatures. Review of publicly available climate charts and published data (Laíns E Silva, 1954; Guerreiro Filho, 1992) shows that both species come from distinctly seasonal environments, with lengthy periods of low monthly rainfall (see also Basic Agronomic Information). In the Inhambane Province in Mozambique, i.e., location of wild and cultivated *C. racemosa*, data from five lowland (<100 m) climate stations shows this species can experience 3–7 months precipitation of <50 mm, and 8–10 months with <100 mm) per annum; the values for the mean annual precipitation were reported between as 682–1,113 mm (Laíns E Silva, 1954). For the same five localities, the following temperatures ranges were reported (Laíns E Silva, 1954): mean maximum temperatures 28.8–30.3°C; mean minimum 9–18.4°C; absolute maximum 33–46°C; and absolute minimum 3–14°C. Temperature ranges for *C. zanguebariae* are likely to be similar, or somewhat warmer based on the modelled mean annual temperature provided by our climate profile analysis. *Ad hoc* absolute maximum temperatures (in shade) of 39–45°C were recorded *in situ* in January and February 2019 (A. Davis pers. observ.) on Ibo and Quirimbas islands in northern Mozambique, using a portable thermo-hygrometer (Tinytag® TH-2500), plants growing nearby were either in shade, partial shade or full sun. Shaded plants appeared to be in better health, had larger leaves, more exuberant growth and more fruits.

Laíns E Silva (1954) remarked that it was surprising that the farming of *C. racemosa* had not been attempted in the Mediterranean region of Europe, given the climate data reported for this species, although mean and absolute low temperatures would probably pose the most obvious constraint to successful cultivation.

Key features for adaptation to this warm, low precipitation, and seasonal environment include deciduous habit, and the short period of fruit development of around 4 months, from flower (i.e., anthesis) to mature (harvestable) fruit. Other key traits are not clearly apparent but may include the short flowering period (individual flowers and total flowering period per plant) and perhaps mature fruit colour (purplish to purple black). Root characteristics (e.g., type, dimensions, architecture, microbiome) may also play a role. Further research in this area would

be worthwhile, as would more direct measurement of above-ground (e.g., temperatures, relative humidity, vapour pressure deficit) and below-ground (soil moisture, soil water potential, temperature) climate metrics.

The rainfall bimodality revealed in the climate profiling analysis for precipitation seasonality for *C. racemosa* (**Table 3**; **Figure 9**) corresponds to the distribution (**Figure 2**) and ecological differences between coastal (< 200 m) and inland populations [200–600(−780 m)]. Whilst this may be interpreted as climate partitioning, with the higher elevation populations experiencing slightly lower mean annual temperature (Bio 1), lower precipitation (Bio 12) and higher precipitation seasonality (Bio 15), as shown in **Table 3**, it is likely the precipitation parameters are offset by the availability of ground water from nearby or adjacent rivers and local environmental features (e.g., adjacency to large rock outcrops) and other microhabitats with higher levels of soil moisture).

Agronomic and Value Chain Considerations

Yields are low for *C. racemosa* and *C. zanguebariae*, compared to the mainstream crop species (*C. arabica* and *C. canephora*). Yields of *C. zanguebariae* appears to be higher than *C. racemosa*, with yields estimated between 300 and 400 g of clean coffee per plant, based on a conversion ratio of 10:1; a planting density of 1,000 plants per hectare would provide 300–400 kg/ha of clean coffee. This is equivalent to lower yield rates of Arabica coffee. At the same conversion ratio and planting density, the yield of *C. racemosa* reported here would give 111 kg/ha, representing a very low yield for commercial coffee, although this is for young and perhaps not fully mature plants. Further yield experimentation is required for both species.

The small seed size of *C. racemosa* and *C. zanguebariae* (**Table 2**; **Figures 4**, **6**) is problematic, due to its contribution to reduced yields, and issues concerned with processing. Small seeds could also be problematic for roasting, as they may require specific equipment and a roasting profile that differs considerably from the main crop species (Arabica and robusta).

Agronomic traits of obvious value for *C. racemosa* and *C. zanguebariae* are extreme heat tolerance, low precipitation tolerance, and extreme precipitation seasonality, i.e., long dry season (number of months with low precipitation values) (**Table 3**; **Figures 8**, **9**). Such attributes would be key to the success of a coffee crop in areas where use of the main production species would be climatically unviable, and could be useful for the production of drought tolerant coffee crop cultivars *via* interspecies breeding. Indeed, *C. racemosa* has been successfully crossed with *C. arabica* (and backcrossed with *C. arabica*) to produce a range of hybrid derived cultivars, notably *C. arabica* cv. 'Siriema' (sometimes referred to more broadly as *C.* 'Aramosa') although crosses have also been made with *C. canephora* (Sureshkumar et al., 2010), and the interspecies hybrid *C.* × 'Congusta' (*C. congensis* × *C. canephora*) (Sureshkumar et al., 2004). These hybrids have been shown to demonstrate a degree of drought tolerance, compared to *C. arabica* (Grisi et al., 2008; Melo et al., 2014; Carvalho et al., 2017) and *C. canephora* (Sureshkumar et al., 2004).

The extremely short fruit ripening period [i.e., the duration from flowering (anthesis) and full fruit ripeness] of around 4 months represent another key trait for *C. racemosa* and *C. zanguebariae*, which has already been investigated by workers. Early ripening plants have been reported for *C. arabica* × *C. racemosa* hybrids, with a 180 day (around 6 months) fruit ripening period, as opposed to a 6–8 month period in *C. arabica* (Sondahl et al., 1997). In hybrids of *C. canephora* × *C. racemosa* a 160–170 (around five and a half months) fruit ripening period has been reported (Sureshkumar et al., 2004, 2010), as opposed to 9–11 months in *C. canephora*.

Originally, the objective of producing hybrids of *C. arabica* × *C. racemosa* was to introduce resistance to coffee leaf miner (*Perileucoptera coffeella*) (Medina Filho et al., 1977b), although *C. racemosa* has also been trialled in breeding work to reduce the caffeine content in *C. arabica* (Medina Filho et al., 1977a).

Flavour

The flavour (aroma and taste) of *C. racemosa* and *C. zanguebariae* is conspicuously distinct from *C. arabica* (Arabica) and *C. canephora* (robusta), mainly due to the presence of unique flavour notes. In particular, many volatile-herbal (e.g., eucalyptus, lavender mint, fir tree), and spice (camphor, cinnamon) notes, and savoury elements immediately set this apart from the regular coffee flavour experience. Many of the tasting notes identified (e.g., jasmine, cinnamon, chocolate, vanilla, blackcurrant) would be accepted as positive flavour attributes for coffee (Spencer et al., 2016); others (e.g., camphor, eucalyptus, savoury, medicinal) probably would be considered as unusual, challenging, and perhaps undesireable. Without doubt, *C. racemosa* and *C. zanguebariae* considerably broaden the sensory envelope of coffee drinking.

CONCLUSION

In this contribution we demonstrated that *C. racemosa* (Inhambane coffee) and *C. zanguebariae* (Ibo coffee, or Zanzibar coffee) are two closely related (sister) species, which are easily differentiated from each other on the basis of their morphology, DNA, and distribution; and to some extent climate profile (annual mean temperature) and flavour. Both species experience high temperatures, low precipitation (rainfall), and distinct precipitation seasonality (a long dry season), in their natural and cultivated environments in East Africa. With a cultivation history of over 200 years, these species are farmed at the distinctly local scale in Mozambique and South Africa, although they may have been grown at greater scale and frequency up until the middle of the last century. Today, they probably represent the world's rarest production coffee species, although many wild *Coffea* species are gathered locally for use as coffee (Davis et al., 2019).

Coffea racemosa and *C. zanguebariae* possess useful traits for coffee crop development, especially under accelerated climate change, due to their tolerance of hight temperatures, low total annual precipitation, and marked precipitation seasonality (long dry season), and short fruit ripening period (around 4

months). In terms of flavour, *C. racemosa* and *C. zanguebariae* are unique, and substantially broaden the sensory experience of coffee drinking. Negative traits for these species include low yield, and small seed (coffee bean) size, and, for some perhaps, their unique flavour notes. Further experimentation is required to more fully understand the climate tolerances of these species, particularly *in situ*, and to understand the underlying mechanisms of adaptation. Further work on sensory assessment, seed (coffee bean) chemistry, and agronomy, is also required.

Coffea racemosa has already been utilized in breeding programmes *via* interspecies hybridization, for pest resistance and drought tolerance, and shortening the duration of fruit development and ripening. *Coffea zanguebariae* has not been utilized in this way, probably due to confusion with other species (e.g., *C. racemosa* and *C. pseudozanguebariae*). The greater robustness (plant and leaf size), higher yield, and larger seed size may provide additional useful traits. The considerable morphological variation of *C. zanguebariae* and *C. racemosa,* particularly for fruit and seed size, and possible flavour diversity, suggests that they would benefit from pre-farm and farm selection for within-species crop improvement.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Genbank DNA sequence repository, accession numbers are provided in **Table 1**.

AUTHOR CONTRIBUTIONS

AD, JM, and IA devised the overall framework of the study. AD undertook the literature survey and herbarium data collection, participated in fieldwork, and was the lead on writing the manuscript. RG undertook the DNA sequencing and analyses and contributed toward the writing of the manuscript. JM led on the climate profiling analyses and contributed toward the writing of the manuscript. AD, IA, and MC undertook the fieldwork and farm survey in Mozambique and CD in South Africa. All the authors devised the elements of the study and contributed data and manuscript refinement.

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Conflict of Interest: CD operates a small farm producing *C. racemosa* in Hluhluwe (South Africa), from where agronomic data was recorded.

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Validating South Sudan as a Center of Origin for *Coffea arabica*: Implications for Conservation and Coffee Crop Improvement

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Cultivated Arabica coffee outside Ethiopia is plagued by low genetic diversity, compromising disease resistance, climate resiliency and sensory potential. Access to the wider genetic diversity of this species may circumvent some of these problems. In addition to Ethiopia, South Sudan has been postulated as a center of origin for Arabica coffee, but this has never been genetically confirmed. We used simple sequence repeat (SSR) markers to assess the genetic diversity of wild and cultivated populations of Arabica coffee from the Boma Plateau in South Sudan, against farmed accessions (of wild origin) from Ethiopia, Yemen, and global cultivars. Our results not only validate Boma Plateau as part of the natural distribution and as a center of origin for Arabica coffee but also indicate that wild populations in South Sudan are genetically distinct from Ethiopian Arabica. This newly identified genetic diversity within Arabica could have the potential for crop improvement through selection and use in breeding programs. Observations and analyses show that the extent and health of the wild population of Arabica in South Sudan have declined. Urgent action should be taken to conserve (*in situ* and *ex situ*) the unique, remaining genetic diversity of wild Arabica populations in South Sudan.

Keywords: Boma Plateau, center of origin, coffee, conservation, genetic diversity, simple sequence repeats (SSR), South Sudan

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INTRODUCTION

Coffee is a globally traded agricultural commodity with an estimated export value of US$ 30 billion in 2019 (http://www.worldstopexports.com/coffee-exports-country/), of which Arabica coffee (*Coffea arabica*) accounts for ∼56% of the total production (International Coffee Organization (ICO), 2021). Despite the documented consumption of this coffee species as a beverage since at least the sixth century, and its contribution to the economy of many countries, it is remarkable that there was no factual data confirming native status in Ethiopia until the twentieth century (Meyer, 1965). The first documented cultivation of Arabica is in Yemen from around the fifteenth century, from where two main genetic types (Bourbon and Typica) were disseminated, giving rise to most of the commercial cultivars of Arabica grown worldwide (Anthony et al., 2002; Scalabrin et al., 2020; Krishnan et al., 2021; Montagnon et al., 2021).

The genetic diversity of farmed Arabica coffee is amongst the lowest of cultivated crops (Scalabrin et al., 2020), although the diversity of farmed (and wild) coffee in Ethiopia has still not been fully explored (Davis et al., 2018). Regardless, crossing global cultivars with cultivated Ethiopian accessions of wild origin has proven to bring significant crop improvement as compared to traditional cultivars (Van der Vossen, 2017; Marie et al., 2020). For example, a new genetic group of cultivated Arabica was revealed recently for Yemen (Montagnon et al., 2021). Additional genetic diversity in the species could be an opportunity for further progress. This is important as the Arabica coffee sector faces serious challenges, including climate change (Davis et al., 2012, 2019, 2021; Bunn et al., 2015; Moat et al., 2017, 2019), coffee leaf rust (*Hemileia vastatrix*) epidemics (Avelino et al., 2015), and the need for new and distinct flavor profiles (Davis et al., 2019; Montagnon et al., 2019).

The first recorded occurrence of *C. arabica* in the Boma Plateau of South Sudan (Anglo-Egyptian Sudan at that time) was in 1929 by A. Chevalier (Thomas, 1942). Following this, J. G. Myers, ecological advisor to the Sudan Government, visited the plateau in 1938 and confirmed *C. arabica* growing wild in the forests. Subsequent to this visit he invited A. S. Thomas, an economic botanist with the Ugandan Department of Agriculture, to accompany him on a second visit in December 1941 to collect seed for breeding purposes (Thomas, 1942). In his paper, Thomas described wild Arabica coffee growing abundantly on the slopes of Nelichu in the two localities of Barbuk and Rume (**Figure 1**). In the Barbuk area, trees growing up to a height of 18 ft. (5.5 m) were documented with much variation in leaves and fruits. Young seedlings were noticed growing near the larger trees as well as beside paths, which were attributed to dropped fruits collected by the local Kichepo tribe. The trees and shrubs listed by Thomas in his account of Barbuk are characteristic of the forests containing wild Arabica coffee in Ethiopia, which are classified as Moist Evergreen Afromontane Forest (MAF), and Transitional Rain Forest (TRF), (Friis et al., 2010). In the Rume area, about 2–3 miles south of Barbuk, coffee was found growing in areas cleared for growing maize (*Zea mays*)—not deliberately planted, but rather relics of the cleared forests. The coffee trees in Rume were shorter than the trees in Barbuk with green-tipped leaves compared to copper-tipped in Barbuk. During this expedition, seeds were collected and sent to the East African Agricultural Research Station in Amani (Tanzania), Coffee Research Station, Lyamungu (Tanzania), Scott Laboratories, Nairobi (Kenya), and various stations in Uganda, and these accessions are likely to be the progenitors of the cultivars *C. arabica* 'Rume Sudan' and 'Barbuk Sudan,' which are in cultivation today. Thomas (1942) noted the bold beans of Barbuk coffee, the relatively low elevation (compared to Barbuk) where the Rume coffee plants coffee were growing, and the lack of noticeable infection by coffee leaf rust (*Hemileia vastatrix*), which were identified as attributes for expanding the useful traits of cultivated Arabica coffee.

There are only a few accessions available in living coffee research collections claimed to be representative of the original wild Arabica collections from the Boma Plateau (Thomas, 1942; Meyer, 1965), although 'Rume Sudan' is cultivated on a few farms as high quality (specialty) coffee, such as in Colombia. In the Centro Agronómico Tropical de Investigación y Enseñanza (CATIE) international coffee collections, two accessions of 'Rume Sudan' and one accession of 'Barbuk Sudan' are available. Records indicate that the two 'Rume Sudan' accessions (T.02724 and T.02744) and the one 'Barbuk Sudan' accession (T.02758) were introduced to CATIE in July 1953 from Kenya and Sudan (this southern part is now South Sudan) respectively. All accessions are documented to have been collected by A. S. Thomas from the Boma Plateau, in 1941. Other than this, the details of their collection are non-existent. No further collection or *in situ* assessment of Arabica from South Sudan has been made since 1941 (Thomas, 1942).

In 2012, as part of a larger team including counterparts from South Sudan, we (SK, APD, TS) undertook fieldwork on the Boma Plateau, which is the only known location of Arabica populations in South Sudan, to collect data of wild and cultivated *C. arabica*. Based on this survey, the objectives of the present study were: (1) to compare the genetic identity of the *C. arabica* from the Boma Plateau against available cultivated accessions from Ethiopia (originally of wild origin), landraces from Yemen, and a selection of Arabica cultivars from around the world; and (2) assess the conservation status of *C. arabica* metapopulation on the Boma Plateau.

MATERIALS AND METHODS

Two genetic studies were conducted: (1) study of all the samples from the field survey of *C. arabica* from the Boma Plateau in South Sudan; and (2) comparison of a subset of South Sudanese samples from Study 1 (South Sudan) with cultivated Ethiopian Arabica (Ethiopian Landraces) and worldwide Arabica cultivar accessions (World Wide Cultivars) from the international coffee collection held at CATIE gene bank, and Yemen landraces from Sana'a University, Yemen.

Plant Material

Study 1: South Sudan Survey

Fieldwork on the Boma Plateau (Upper Boma, Jonglei State, South Sudan) was conducted 9–12 April 2012. **Figure 1** shows the area covered by the expedition, during four days of fieldwork. Collections were made from cultivated and wild plants in local villages (Rumit/Zoch, Kaiwa, Jonglei and Bayen) and two forest locations (Rumit and Ngelecho), respectively. A total of 74 samples were collected and used in this study. **Table 1** provides a summary of the cultivated and wild accessions sampled. In addition to the samples collected in South Sudan, two domesticated *C. arabica* accessions cultivated at Denver Botanic Gardens (DBG) were added to the study as controls. An accession list is provided in **Supplementary Table 1**. For Study 1, since sampling was from the field collections in South Sudan, we were restricted by the number of trees we were able to access in the forests, leading to unequal sample sizes.

Location coordinates were recorded using a Garmin eTrex Vista NCx and a Holux M-241. Several leaves of each individual plant were collected and placed in plastic bags with silica gel (Chase and Hillis, 1991). Voucher specimens of selected samples were collected in replicates of three, and are housed at the

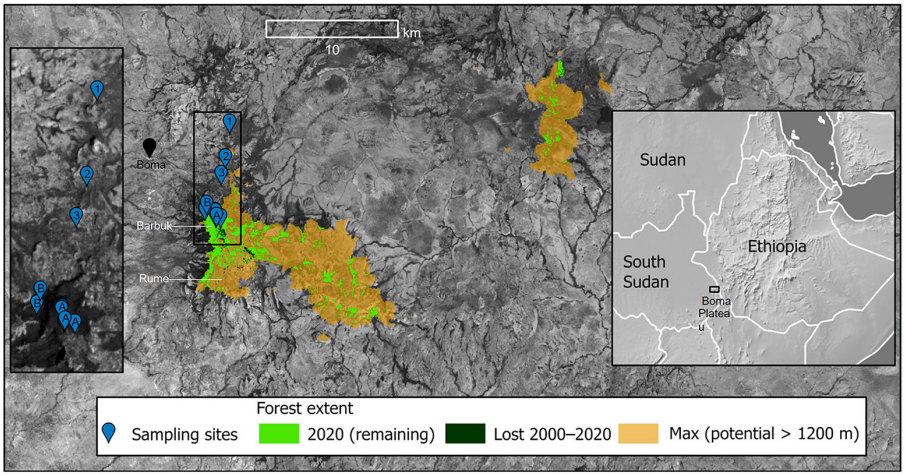

FIGURE 1 | Study area: Boma Plateau of South Sudan. Cultivated collecting sites: 1 = Bayen; 2 = Jonglei; 3 = Kaiwa. Wild collecting area: A = Rumit (Barbuk), on plateau [includes some cultivated plants (see text)]; B = Ngelecho (Barbuk), on escarpment. Inset map right shows regional location of study area; inset map left shows location detail of main sampling sites.

TABLE 1 | Summary of *Coffea arabica* populations sampled in South Sudan and used in studies 1 and 2.

Population name	Collection numbers*	#Samples study 1	#Samples study 2
Rumit cultivated 1	B1–B32	32	5
Rumit cultivated 2	B47–B56	10	3
Kaiwa and Jonglei cultivated	B57; B59	2	2
Bayen cultivated	B61–B63	3	3
Rumit wild	B33–B46	14	5
Ngelecho wild	B64–B76	13	5
Cultivars held at Denver botanic gardens collection (DBG), (control)	B77–B78	2	2
TOTAL		**76**	**25**

Detailed collections list with herbarium sample information is provided in **Supplementary Table 1.*

TABLE 2 | Summary of *C. arabica* samples used in study 2 for comparing South Sudanese samples with worldwide collections.

Category*	#Samples
Ethiopian landrace	116
Worldwide cultivar	34
Yemen landrace	10
South Sudan survey	20
Total	**180**

Detailed collections list is provided in **Supplementary Table 2.*

herbaria at the Royal Botanic Gardens, Kew (K) and Missouri Botanical Garden (MO), with the third replicate destined for a suitable location in South Sudan (herbarium not yet established). As collections were being made, individual trees were tagged with numbered aluminum tags nailed to the ground, and with orange tree tape with the corresponding number tied to the truck of the tree. Tagging was done for ease of location and plant identification for subsequent seed collecting expeditions.

Study 2: Genetic Comparison of South Sudan Samples With Ethiopian and Worldwide Collections

For this study, we used 180 samples. Following the identified groupings and nomenclature of Scalabrin et al. (2020), Pruvot-Woehl et al. (2020), and Montagnon et al. (2021), the following categories were considered (**Table 2**):

- Ethiopian landraces: 116 accessions (all from CATIE)
- Worldwide cultivars: 34 accessions (which included 5 accessions from the South Sudan survey and 29 accessions from CATIE)
- Yemen landraces: 10 accessions
- South Sudan wild populations: 20 accessions

The accessions from the CATIE collection are predominantly from the germplasm collecting missions undertaken by the Food and Agriculture Organization (FAO, 1968) and the French organization ORSTOM—Office de la Recherche Scientifique et Technique Outre-Mer (now known as Institute de Recherche pour le Développement—IRD), (Charrier, 1978).

This sampling subset was designed to understand the genetic diversity of coffee on the Boma Plateau, which is potentially the last remaining stronghold of Arabica diversity in South Sudan, and how it compares with those in collections worldwide. Hence, the sample sizes are unequal. Based on the genetic assignment from Study 1, the 25 South Sudan subsamples were allocated as 20 to South Sudan Survey and five to Worldwide cultivars (**Table 2**). The samples used in Study 2 are provided in **Table 2**; details of these collections are provided in **Supplementary Table 2**.

TABLE 3 | List of microsatellite markers used in study 1 with their locus code, primer sequences, and product size.

Locus code	Primer sequence	Product size (bp)	Reference
M254	F: GGCTCGAGATATCTGTTTAG R: TTTAATGGGCATAGGGTCC	132	Combes et al., 2000
M255	F: CCCTCCCTGCCAGAAGAAGC R: AACCACCGTCCTTTTCCTCG	160	Combes et al., 2000
M256	F: AGGAGGGAGGTGTGGGTGAAG R: AGGGGAGTGGATAAGAAGG	118	Combes et al., 2000
M257	F: GACCATTACATTTCACACAC R: GCATTTTGTTGCACACTGTA	103	Combes et al., 2000
M258	F: AACTCTCCATTCCCGCATTC R: CTGGGTTTTCTGTGTTCTCG	100–132	Combes et al., 2000
M259	F: ATCCGTCATAATCCAGCGTC R: AGGCCAGGAAGCATGAAAGG	72	Combes et al., 2000
M260	F: TGATGGACAGGAGTTGATGG R: TGCCAATCTACCTACCCCTT	100	Combes et al., 2000
M746	F: GGCCTTCATCTCAAAAACCT R: TCTTCCAAACACACGGAGACT	378	Rovelli et al., 2000
M764	F: CTGGCATTAGAAAGCACCTTG R: GCTTGGCTCACTGTAGGACTG	158	Rovelli et al., 2000
M774	F: GCCACAAGTTTCGTGCTTTT R: GGGTGTCGGTGTAGGTGTATG	228	Rovelli et al., 2000
M779	F: TCCCCCATCTTTTTCTTTCC R: GGGAGTGTTTTTGTGTTGCTT	116	Rovelli et al., 2000
M780	F: ATTCTCTCCCCCTCTCTG R: GTTAGTATGTGATTTGGTGTGG	95	Rovelli et al., 2000
M782	F: AAAGGAAAATTGTTGGCTCTGA R: TCCACATACATTTCCCAGCA	114	Rovelli et al., 2000
M784	F: TTGCTTGCTTGTTCTGTTAT R: TGACACGAGAGTTAGAAATGA	126	Rovelli et al., 2000
M790	F: TTTTCTGGGTTTTCTGTGTTCTC R: TAACTCTCCATTCCCGCATT	134	Rovelli et al., 2000
M809	F: AGCAAGTGGAGCAGAAGAAG R: CGGTGAATAAGTCGCAGTC	144	Rovelli et al., 2000
M837	F: CTCGCTTTCACGCTCTCTCT R: CGGTATGTTCCTCGTTCCTC	102	Rovelli et al., 2000
M838	F: CCCGTTGCCATCCTTACTTA R: ATACCCGATACATTTGGATACTCG	100	Rovelli et al., 2000
M883	F: CGTCTCGTTTCACGCTCTCT R: GATCTGCATGTACTGGTGCTTC	237	Rovelli et al., 2000

DNA Extraction and Molecular Markers

Study 1: South Sudan Survey

Genomic DNA for Study 1 was extracted from 10 mg of silica-dried leaf material using GenCatch™ Plant Genomic DNA Purification kits (Epoch Biolabs) at the Conservation Genetics lab at Denver Botanic Gardens. Slight modifications were made to the extraction protocols: a detailed account of the extraction procedure is described in Krishnan (2011). Extracted DNA was sent to Nevada Genomics, Reno, Nevada, USA, for quantification, optimization, fragment analysis, and scoring using microsatellite markers. Initially, 20 microsatellite markers were selected based on Combes et al. (2000), Rovelli et al. (2000), Poncet et al. (2004), and Cubry et al. (2008). These markers were selected due to their high polymorphism in *C. arabica*. Of these 20 markers, one did not amplify (M253), and so this marker was dropped from the study. The remaining 19 markers were used (**Table 3**).

The DNA was quantified and normalized to 5 ng/μl. PCR amplifications were conducted using an MJ thermocycler. Each 10 μl PCR amplification reaction contained 4 μl of 5 ng/μl genomic DNA, 1 μl Primer Panel mix, and 5 μl Qiagen Multiplex PCR Mix. The 19 microsatellite markers were multiplexed in four panels with a different annealing temperature for each panel. The PCR cycling conditions were as follows: 95°C for 15 min; 95°C for 30 s; specific panel annealing temperature for 45 s; 40 cycles at 72°C for 45 s; 72°C for 30 min. Panel 1 consisted of M780, M790, M259, M254, and M257 with an annealing temperature of 60°C. Panel 2 consisted of M258, M746, M782, M837, and M784 with an annealing temperature of 61°C. Panel 3 consisted of M256, M838, M255, M809, and M883 with an annealing temperature of 63°C. Panel 4 consisted of M260, M779, M774, and M764 with an annealing temperature of 62°C. The samples were run on an Applied Biosystems Prism 3730 DNA Analyzer. The filter set used was G5, which detects the fluorescent dyes 6-FAM, VIC, NED, and PET. The samples were run with the 500 MW size standards labeled with LIZ. The fragment analysis results were scored using GeneMarker 2.2.0 Software by SoftGenetics®.

Study 2: Genetic Comparison of South Sudan Samples With Ethiopian and Worldwide Cultivar Collections

DNA extraction and SSR marker analysis were performed by the ADNiD laboratory of the Qualtech company in the South of France (http://www.qualtech-groupe.com/en/). Genomic DNA was extracted from ∼20 mg of dried tissue using 1 ml of SDS buffer. DNA was then purified with magnetic bead (Agencourt AMPure XP, Beckman Coulter, Brea, California, USA) followed by elution in Tris Edta (TE) buffer. The DNA concentration was estimated with an Enspire spectrofluorimeter (Perkin Elmer) with a bisbenzimide DNA intercalator (Hoechst 33258) and by comparison with known standards of DNA.

Eight SSR primer pairs (**Table 4**) selected after Combes et al. (2000) and whose wide discrimination power was confirmed by Pruvot-Woehl et al. (2020) were used. This reduced set of markers were demonstrated to be efficient. PCR was performed in a 15 μl final volume comprising 30 ng genomic DNA and 7.5 μl of 2× PCR buffer (Type-it Microsatellite PCR Kit, Qiagen), 1.0 μM each of forward and reverse primer (10 μM). Amplifications were carried out in thermal cycler (Eppendorf) programmed at 94°C for 5 min for initial denaturation, followed by 94°C for 30 s, annealing temperature depending on the primer used for 30 s and 72°C for 1 min for 35 cycles followed by a final step of extension at 72°C for 5 min. The final holding temperature was 4°C. PCR samples were run on a capillary electrophoresis, ABI 3130XL with an internal standard: GeneScan 500 LIZ size standard (Applied Biosystems). Alleles were scored using GeneMapper v.4.1 software (Applied Biosystems) and then visually inspected.

TABLE 4 | List of microsatellite markers used in study 2 with their locus code, primer sequences, and product size.

Locus code	Primer sequence	Product size (bp)
Sat11	F: ACCCGAAAGAAAGAACCAA R: CCACACAACTCTCCTCATTC	143–145
Sat24	F: GGCTCGAGATATCTGTTTAG R: TTTAATGGGCATAGGGTCC	167–181
Sat29	F: GACCATTACATTTCACACAC R: GCATTTTGTTGCACACTGTA	137–154
Sat32	F: AACTCTCCATTCCCGCATTC R: CTGGGTTTTCTGTGTTCTCG	119–125
Sat47	F: TGATGGACAGGAGTTGATGG R: TGCCAATCTACCTACCCCTT	135–169
Sat225	F: CATGCCATCATCAATTCCAT R: TTACTCCTCATCATTCCGCA	283–317
Sat235	F: TCGTTCTGTCATTAAATCGTCAA R: GCAAATCATGAAAATAGTTGGTG	245–278
Sat254	F: ATGTTCTTCGCTTCGCTAAC R: AAGTGTGGGAGTGTCTGCAT	221–237

TABLE 5 | Clustering of South Sudan survey populations.

Populations	Clusters (number of plants per cluster)			Total
	1	2	3	
Ngelecho wild	11	2		**13**
Rumit wild	2	12		**14**
Rumit cultivated 1	18	14		**32**
Kaiwa and Jonglei cultivated	1	1		**2**
Rumit cultivated 2	4	6		**10**
Bayen cultivated			3	**3**
DBG collection			2	**2**
Total	**36**	**35**	**5**	

Data Analysis

Study 1: South Sudan Survey

To identify genetic clusters in populations, DARwin6 software (Perrier and Jacquemoud-Collet, 2006) was used with single data files. The data were entered into a binary matrix as discrete variables, one for presence and zero for absence of the character. The dissimilarity matrix was calculated using Dice Index and was the basis for the execution of the Principal Coordinates Analysis (PCoA). PCoA provides an overall representation of the diversity (Perrier and Jacquemoud-Collet, 2006) and produces graphical representations on Euclidean plans which preserve the distances between units.

Study 2: Genetic Comparison of South Sudanese Samples With Ethiopian and Worldwide Cultivar Collections

All the genotypes were scored for the presence and absence of the SSR bands. The data were entered into a binary matrix as discrete variables, one for presence and zero for absence of the character and this data matrix was subjected to further analysis. Discriminant Analysis of Principal Components (DAPC) was performed to identify and describe clusters of genetically related individuals using the adegenet package version 2.1.3. The optimal number of clusters was determined using the function "find.clusters" which applies successive K-means clustering. The bayesian information criterion (BIC) was visually examined to define the number of clusters. Then, the "dapc" function was applied. The graphs were designed using ggplot2 package Version 3.3.2. PCA-based clustering was also done using the subroutine EIGEN.

Study 3: Conservation Analyses

The aims of the conservation analyses were to determine the current extent of *C. arabica* habitat on the Boma Plateau, compared to a potential maximum, and measure recent forest loss (2000–2020). The suitable forest types for *C. arabica* on the Boma Plateau are the same as that in neighboring Ethiopia, i.e., Moist Afromontane Forest (MAF) and Transitional Rain Forest (TRF), [found at 650–2,600 m (450–3,000 m including extremes)], although coffee is mostly confined to 1,200–2,200 m (Moat et al., 2017). To achieve our objectives for this part of the study we used Google Earth Engine (Gorelick et al., 2017) to query high-resolution elevation data (Jarvis et al., 2008), tree cover for 2000 and global forest change from 2000–2020 (Hansen et al., 2013). For our maximum forest cover we used a conservative minimum elevation threshold of 1,200 m [as per our observations and those of Thomas (1942)], for forest cover at the year 2000, with a tree cover of 50% or more. Forest cover for 2020 was calculated by removing the area of deforestation as identified by the updated dataset from Hansen et al. (2013).

RESULTS

Study 1: South Sudan Survey

Cluster analysis following PCoA revealed three population clusters (**Table 5**; **Figure 2**). The first cluster consisted mainly of plants growing wild in the forests of Ngelecho while the second cluster consisted of plants growing wild in the forests of Rumit. The third cluster consisted of cultivated plants from DBG (control) together with plants cultivated at Bayen village. Two samples from Ngelecho wild population fell into cluster 2 and two samples from the Rumit wild population fell into cluster 1. Plants cultivated in the village of Rumit (identified as two different populations based on distance between populations, **Table 1**; **Figure 2**) were split between clusters 1 and 2, with 22 plants falling in cluster 1 (Ngelecho Wild) and 20 plants falling in cluster 2 (Rumit Wild). The one plant cultivated at Kaiwa village clustered with Ngelecho Wild (cluster 1) and the one plant from Jonglei village clustered with Rumit Wild (cluster 2). Cluster 3 identified as plants belonging to cultivated and introduced variants consisted of the three plants collected at Bayen village and the two plants from the collections at Denver Botanic Gardens (DBG). Since the Bayen population was closely related to cultivated plants, for the subsequent study (Study 2), these

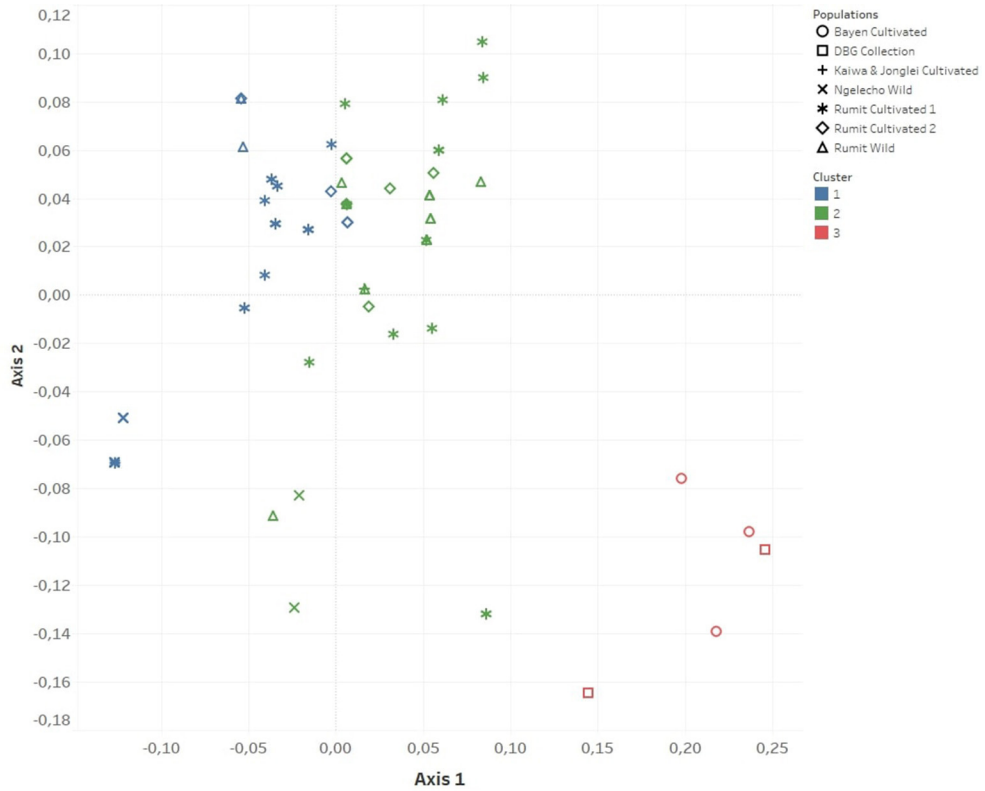

FIGURE 2 | Clustering of South Sudan survey populations (Study 1) on a graph on the first two axes of the Principal Coordinates Analysis (PCoA) based on dissimilarity matrix.

five plants (Bayen and DBG) were allocated to the Worldwide Cultivar category.

Study 2: Genetic Comparison of South Sudanese Samples With Ethiopian and Worldwide Cultivar Collections

The 180 samples used in this study were partitioned into six clusters after DAPC. One group was only made of South Sudan accessions, which we named "South Sudan." Three clusters were made of Ethiopian accessions only; we named them EL1, EL2, and EL3. Only Worldwide Cultivar SL-06 was in EL3 cluster. Two other clusters included some Ethiopian accessions, some Yemen Landrace and some Worldwide cultivars. We named these clusters WWC1 and WWC2 (**Table 6**; **Figure 3**).

The South Sudan group had one unique allele (allele 154 of Sat24). Another allele (allele 127 of Sat 47) had a frequency of 1.00 in the South Sudan group and a frequency of 0.00 in all other groups except WWC2 where the frequency was 0.10. In WWC2, this allele was borne by 3 accessions: one of the "Rume Sudan" accessions (CATIE code: T.02724), SL-14 (T.02747) and SL-17 (T.02745). The two other supposed South Sudanese accessions from CATIE did not bear any of these two alleles and were also part of the WWC2 cluster: "Rume Sudan" (T.02744) and "Barbuk Sudan" (T.02758), (Data not shown), (**Figure 3**; **Supplementary Table 2**).

Study 3: Conservation Analyses

The total suitable forest cover for *C. arabica* in the Boma Plateau area for 2020 was 15.3 km^2, compared to 16.2 km^2 in 2000, representing a forest cover loss of 5.8% over 20 years (**Figure 1**). The maximum potential forest cover suitable for *C. arabica* (i.e., humid forest above 1,200 m) for the Boma Plateau area was calculated to be 97 km^2, which would represent a potential forest loss of 81.7 km^2, i.e., a loss of 84.2%. The wild populations of *C. arabica* on the Boma Plateau are 88 km from the nearest metapopulation in Ethiopia (around Geba); satellite data also show that this forested area is also being rapidly lost (Hansen et al., 2013).

If we use these data for a regional/country-level, by applying International Union for the Conservation of Nature (IUCN) Red List Categories and Criteria (IUCN Standards Petitions Subcommittee, 2017), the Area of Occurrence (AOO) of 15.3 km^2 would place it into the Endangered category (EN), approaching the Critically Endangered category (CR). However, under climate change (Davis et al., 2012; Moat et al., 2019) the Boma Plateau area will lose climate suitability for *C. arabica* by 2010–2039. This would push a regional assessment to CR using a conservative generation length of under 6 years (Moat et al., 2019 recommend a generation length of 21 years for *C. arabica*).

TABLE 6 | Clustering of populations identified as Ethiopian landrace, worldwide cultivar, Yemen landrace and South Sudan survey.

	Cluster definition						
Category	**South Sudan**	**EL1**	**EL2**	**EL3**	**Worldwide cultivar 1 (WWC1)**	**Worldwide cultivar 2 (WWC2)**	**Total**
Ethiopian landrace		19	42	35	10	10	116
Worldwide cultivar				1	17	16	34
Yemen landrace					6	4	10
South Sudan survey	20						20
Total	**20**	**19**	**42**	**36**	**33**	**30**	**180**

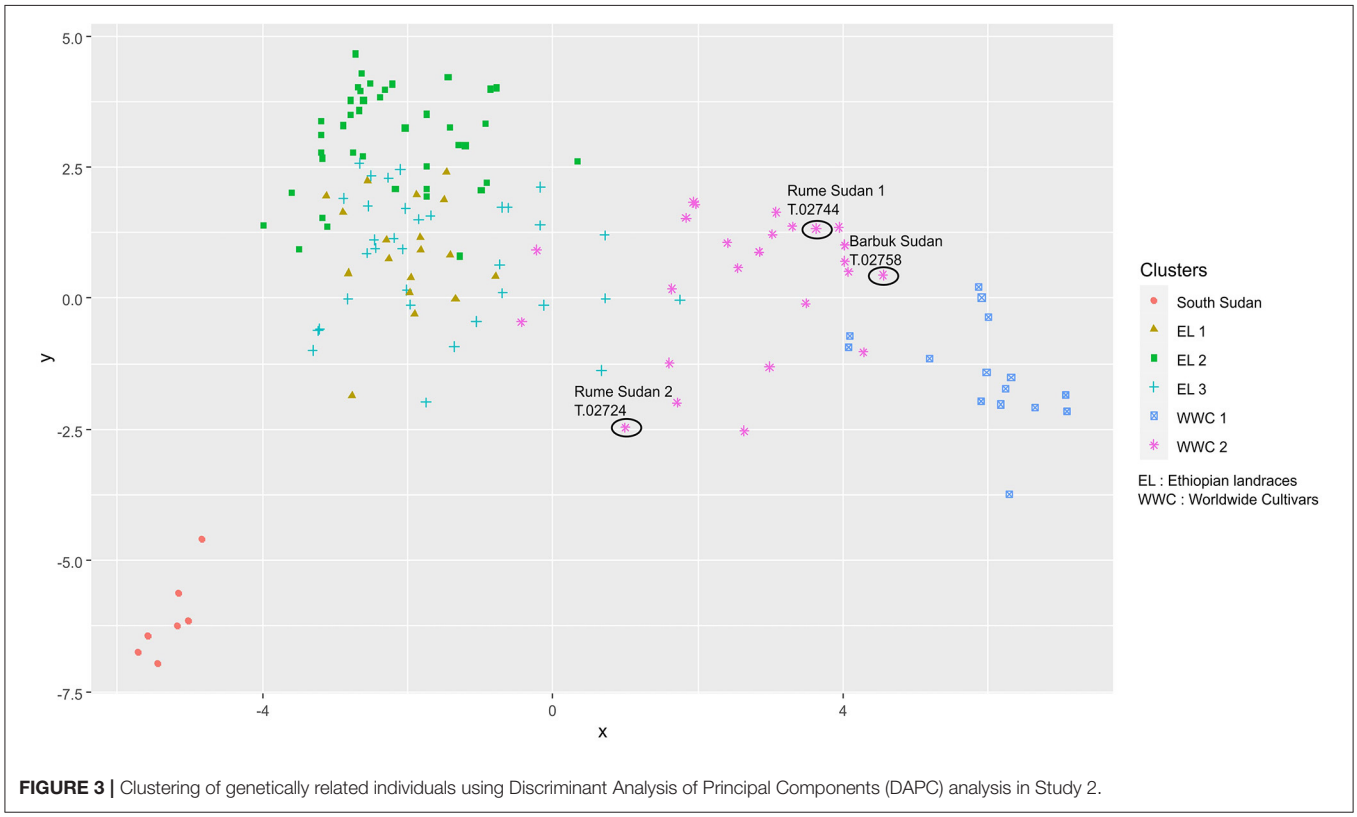

FIGURE 3 | Clustering of genetically related individuals using Discriminant Analysis of Principal Components (DAPC) analysis in Study 2.

DISCUSSION

Genetic Diversity of South Sudan Arabica

The unique outcome of this study is the identification of the metapopulation of wild Arabica on the Boma Plateau (South Sudan) as a genetically separate entity, distinct from Ethiopian landraces, global Worldwide Cultivars and Yemen landraces. Thomas (1942) suggested that the *C. arabica* growing on the Boma Plateau (at Barbuk, Rume and Nelichu) could have been brought for cultivation from Abyssinia (present-day Ethiopia). He reasoned that the Boma Plateau and the adjacent wild coffee forests of the Ethiopian Highlands were separated by low, open woodland (mostly *Combretum-Terminalia* Woodland and Wooded Grassland (Friis et al., 2010) unfavorable for the growth of *C. arabica* (Davis et al., 2012). However, the distance between the Boma Plateau and suitable forested areas (even based on current forest cover and climate) is only 80 km, and pre-Anthropocene there would have been numerous suitable forest patch "stepping stones" between Boma and the Ethiopian Highlands. These observations argue for a natural distribution across the Ethiopian Highlands and a small area of eastern South Sudan (including the Boma Plateau). There is certainly some disjunction between the Boma Plateau and Ethiopian populations, but this also is the case with the Ethiopian Highlands (Davis et al., 2018).

The two wild populations collected from Boma Plateau, Ngelecho Wild and Rumit Wild were distinct from each other, forming separate clusters. The plants cultivated in nearby villages were a mix of both populations. Thomas (1942) documented young coffee plants growing beside the paths just inside the edge of the forest and noted that these probably arose from fruits dropped by the Kichepo from harvests made in the forests. This movement of harvested fruits from the forests could account for the presence of both wild populations among the two cultivated populations sampled.

Our current study shows the coffee growing in the Boma Plateau of South Sudan to be genetically distinct from that collected in Ethiopia by the FAO and ORSTOM missions in the 1960s and those in cultivation, and that there is strong support for South Sudan as the center of origin of Arabica. This extension of the genetic diversity for Arabica is important, given that until now Ethiopia was the only established origin of diversity, and that the genetic diversity of cultivated Arabica outside Ethiopia is desperately low (Scalabrin et al., 2020). Other key outcomes are given in the following narrative.

The CATIE collection houses three accessions recorded as being collected in Sudan by Thomas (1942): two 'Rume Sudan' (T.02724 and T.02744) and one 'Barbuk Sudan' (T.02758) accessions. Genebank records indicate that the 'Rume Sudan' and 'Barbuk Sudan' accessions were introduced to CATIE in July 1953 from Kenya. The Scott Agricultural Laboratories in Kenya and Lyamungo Research Station in Tanganyika became the main centers for coffee breeding in East Africa (Van der Vossen, 1985), both of which received the seed of 'Rume Sudan' and 'Barbuk Sudan' from the Thomas expedition in 1941. Passport data indicate the two 'Rume Sudan' accessions collected in Rume region of Boma Plateau and the 'Barbuk Sudan' collected in the Barbuk region of Boma Plateau in 1941 by A. S. Thomas. There is also documentation of 'Rume' and 'Barbuk Sudan' accessions in the USDA collections dating to 1953 at Glendale, Maryland and 1957 at Puerto Rico, though these collections do not exist at the current time (M. Winterstein, personal communication). The two 'Rume Sudan' and one 'Barbuk Sudan' cultivars from the CATIE collection however did not group with the South Sudan samples. These accessions clustered with the Worldwide Cultivar group (**Figure 3**). However, 'Rume Sudan' (T.02724) possesses one of two alleles that are specific to the South Sudan group. Hence, this 'Rume Sudan' accession may have originated from a true 'Rume Sudan' population but cross-pollinated with other Arabica accessions in germplasm collections where they were held before being introduced to CATIE gene bank. The other two accessions, one 'Rume Sudan' and one 'Barbuk Sudan' have either been more diluted through cross pollination or perhaps mislabeled in the CATIE collection or previous germplasm collection. There is also the possibility that the 'Rume Sudan' and 'Barbuk Sudan' currently in cultivation as well as in global gene banks are possibly genetically contaminated from growing in the Scott Agricultural Laboratories research stations with other cultivars, or plants distributed globally from Kenya and Tanzania could have been mislabeled. Hence, future research needs to be undertaken to genotype all Rume and Barbuk Sudan in gene banks and farmers' fields to track the original collections made by Thomas. Two other accessions of the CATIE germplasm collection had one of the two specific alleles to the South Sudan group: SL-14 (T.02747) and SL-17 (T.02745). Interestingly, these two accessions represented one specific mother population (named 'SL-17') in the study by Montagnon et al. (2021). The accessions that are part of that mother population do not seem to have followed the Yemeni domestication route. It might well be that this SL-17 mother population is somehow related to South Sudanese accessions.

There were no clear geographical repartition patterns between the three Ethiopian groups (EL1, EL2 and EL3). The Ethiopian accessions used in our study were originally from the FAO (1968) and ORSTOM (Charrier, 1978) coffee surveys, which mostly covered coffee in cultivation and wild coffee forest areas to the west of the Rift Valley, in the South West and Rift coffee zones (Davis et al., 2018). Furthermore, the Ethiopian accessions in our study are part of the core collection established by World Coffee Research (WCR) and CATIE. Scalabrin et al. (2020) included the more than 500 Ethiopian accessions held by CATIE in their study, using SNPs, but only found a weak population structure with a slight West to East geographical cline. Despite this large sample size, no studies have yet comprehensively sampled the entire geographical range of wild and cultivated coffee areas in Ethiopia. Notable exceptions, where either no or very few accessions have been sampled, include the coffee areas west of the Rift Valley, in Amhara, Wellega, northern Illubabor, and Bench Maji; and East of the Rift Valley, in Sidamo, Bale, Central Eastern Highlands, Arsi, West Hararge and East Hararage (Davis et al., 2018). The wild Arabica populations in the forest of western Wellega, northern Illubabor, western Bench Maji and Bale (Davis et al., 2018) will no doubt be found to represent key centers of diversity using molecular data and may help to elucidate the genetic-geographical structure of wild Arabica in Ethiopia. Consistent with other studies (Pruvot-Woehl et al., 2020; Scalabrin et al., 2020; Montagnon et al., 2021), SL-06 was found to be included in an Ethiopian cluster. We therefore suggest to include this accession in the Ethiopian Landrace accessions, instead of in the Worldwide Cultivars. There was no clear interpretation of the two Worldwide Cultivars genetic groups. This is not surprising, however, as only by adding more diverse samples from Yemen were Montagnon et al. (2021) able to give a complete description of the genetic structure of the worldwide Arabica coffee cultivars.

Former studies (Anthony et al., 2002; da Silva et al., 2019; Benti et al., 2020) had proven the efficiency of SSR markers to study the genetic diversity of Arabica. In our study, we confirm that the reduced set of SSR markers established by Pruvot-Woehl et al. (2020) are efficient to describe a new genetic group in Yemen (Montagnon et al., 2021) is again efficient to describe a new genetic group in this species: the South Sudan group. Scalabrin et al. (2020) found the vast majority of the SNPs found were of low-frequency alleles. Recently, apparently more workable SNPs were published (Zhang et al., 2021); testing these SNPs on the South Sudan samples might be a good way to confirm their accuracy.

The three cultivated trees from Bayen village did not cluster with the other two wild South Sudanese clusters. Instead, they clustered with the cultivated trees from the collections at Denver Botanic Gardens, suggesting that these were more closely related to cultivated genotypes. In the second study comparing the South Sudanese survey collections with Ethiopian, Yemeni and Worldwide Cultivars, two of the Bayen trees were identified within the WWC genetic groups. The inference made from this is that the Bayen Cultivated population is of a different origin, and probably representing introduced cultivated material. Discussion with several individuals from the local village in Upper Boma revealed that in the late 1940s, the British had

attempted coffee cultivation in this area and may have introduced foreign germplasm at that time.

During the 2012 expedition, even though we did not observe coffee being harvested and used commercially, the observation of cultivated trees in Rumit, Kaiwa and Jonglei villages clearly point to the use of coffee by the villagers. The wild coffee from the forests of Rumit and Ngelecho formed two genetic clusters with limited admixture (**Table 5**). The cultivated coffee in Rumit village consisted of a mix of Rumit wild and Ngelecho wild genotypes indicating collections made from both forests by the local villagers. The single tree in Kaiwa village had been collected from the Ngelecho forest whereas the single tree in Jonglei village has been collected from the Rumit forest (**Supplementary Table 2**).

Conservation of South Sudan Arabica

We demonstrate that the humid forests (MAF and TRF; Friis et al., 2010) of the Boma Plateau, where *C. arabica* exists as a wild plant, have experienced large-scale deforestation. Even in the 1940s it was noted (Thomas, 1942) that the natural forests of Boma Plateau had experienced a long history of human disturbance. Compared to what would be expected prior to human intervention (potential forest cover) we estimate an 81.7 km^2 (84.2%) loss of forest cover, and over the last 20 years an 0.9 km^2 (5.8%) loss of forest cover. However, during our field survey in 2012 we observed that even in areas with more than 50% forest cover, the understory of the forest is largely unsuitable for the existence of spontaneous wild *C. arabica* in many places, due to understory clearance, particularly on the forested plateau. At Barbuk (**Figure 1**) the understory vegetation has been largely removed or degraded, with evidence of widespread human disturbance, including ephemeral habitation. Some of the canopy trees were in poor health, especially the emergent canopy *Manilkara butugi* (Sapotaceae), with some standing dead trees. At the edges of Barbuk, on the escarpment (western edge of plateau; **Figure 1**), there was evidence of fire encroachment (i.e. a charcoal layer under the leaf litter), due to the seasonal burning of the surrounding *Combretum-Terminalia* woodland/grassland. At Barbuk we found very few wild *C. arabica* trees, which were of limited age classes (only small trees), and there were few or no seedlings. Our observations are thus in stark contrast to those made by Thomas (1942). It is clear from Thomas (1942) that when he visited Barbuk the forest was in good condition, as he states: "At Barbuk there was a large area of closed forest at an altitude of about 4,700 ft. (1,350 m). The forest was dense, with an evergreen canopy of larger trees"… "Lianes were abundant. There was a thick undergrowth of shrubs …." Thomas (1942) observed wild *C. arabica* as being "locally frequent," with the presence of many mature trees, and plentiful seedlings. The difference between the two surveys are no doubt due to the change in forest health at this locality, but also perhaps because nearby villagers collect *C. arabica* coffee leaves for the production of coffee leaf tea (Campa et al., 2012).

The forested area at Rume (**Figure 1**) was already largely deforested when visited by Thomas in 1941 (Thomas, 1942). He states: "Until 4 or 5 years ago the valley had been with forest, but since the Italian invasion of Abyssinia much clearance and settlement had taken place. At the time of our visit many trees remained; some had fallen but had not yet rotted; others were still alive"… "After clearing, the land had been planted with maize which, together with considerable amounts of small-leaved tobacco, covered the valley floor."… "Standing out dark among the ripening maize were scattered bushes of *Coffea arabica*, either single or in groups. We were told that none of these bushes had been planted and that they were relicts of the original undergrowth of the forest, retained when the other species were cut and burnt." Thomas reports a point elevation for Rume of 4,100 ft. (1,250 m), which is supported by satellite data (GoogleEarth®), and thus generally of lower elevation than Barbuk (see above). The generally flatter topology or Rume may have either made it easier for clearance or made it more suitable for agriculture, and probably both. When visiting in 2012 there was no remaining forest at Rume, although we did not examine the area for remnant *C. arabica* trees within either the cultivated areas or small patches of secondary vegetation. Satellite imagery confirms these observations (**Figure 1**). *C. arabica* is intolerant of disturbance (Davis et al., 2012; Moat et al., 2019) and after forest removal rapidly declines and disappears.

Overall, observations made in the most suitable areas on the Boma Plateau indicated that the *C. arabica* populations are in poor health (loss of aged individuals, meager population density, zero or minimal seedling recruitment, low ratio of flower bud development) compared to 70 years ago (Thomas, 1942).

It is unlikely that any *C. arabica* plants exist now at Rume. If all cultivated accessions of *C. arabica* 'Rume' are compromised, as found in the samples we used in our SSR survey, the genetic diversity from Rume may no longer exist in its original form, if at all. The net result of all anthropogenic influences has been the reduction in the range, density and health of *C. arabica* populations on the Boma Plateau, which has no doubt resulted in a loss of their genetic diversity.

A country-level conservation assessment for *C. arabica* in South Sudan, when applying the Red List Categories and Criteria (IUCN Standards Petitions Subcommittee, 2017), returns a threat level of Endangered (EN), with the major threats being deforestation and understory clearance. When factoring in climate change (Moat et al., 2019) the threat level would rise to Critically Endangered (CR). Climate change projection analyses show that climatic suitability for *C. arabica* on the Boma Plateau (and therefore this species in South Sudan) would cease to exist between 2010 and 2039 (Davis et al., 2012; Moat et al., 2019).

Implications for Conservation and Utilization of South Sudanese Germplasm for Coffee Crop Improvement

This study reveals that in addition to Ethiopia, the Boma Plateau of South Sudan is a center of origin of *C. arabica,* and supports the assumption that it is part of the natural range of this species (Thomas, 1942; Davis et al., 2012). During his 1941 expedition, Thomas (1942) observed significant phenotypic variation in the coffee plants as well as a lack of infection by coffee leaf rust (Thomas, 1942). Partial resistance to coffee leaf rust in Boma Arabica has since been documented (Van der Vossen, 1985)

and South Sudan Arabica germplasm has been used in breeding programs (Marie et al., 2020). Its sensory qualities are appreciated by the specialty coffee sector. These attributes provide useful resources for breeding and utilization in crop improvement programs for Arabica coffee. Our 2012 expedition was able to verify that Arabica coffee still exists in the forested area of Barbuk, where it was found growing wild by Thomas (1942), but also show that the populations there today are in poor health. Field observation in 2012 and satellite imagery (**Figure 1**) show that the second main location on Boma Plateau, i.e., Rume, is now in a deforested state, although even in 1941 this area was already mostly deforested (Thomas, 1942). Over the last several decades the impact of human activities, and possibly climate change, has had a major impact on the extent and health of the Boma Plateau populations. Given that the Boma populations represent a distinct genetic entity for Arabica diversity, and that there is still substantial genetic diversity within the Boma metapopulation, it is imperative that all attempts be made to conserve this diversity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SK, AD, and TS conducted the expedition to South Sudan. SK conducted the genetic diversity studies of South Sudan samples, following which SP-W and CM compared the genetic diversity of South Sudan samples with accessions from CATIE and Yemen provided by WS and AA respectively. JM and AD conducted the conservation assessment. SK took the lead on writing the manuscript with participation from SP-W, CM, and AD. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.761611/full#supplementary-material

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A Methodological Approach for Prioritization and Rationalization of Field Genebank Accessions of Coffee Genetic Resources: A Case Study of CATIE International Coffee Collection, Costa Rica

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Good management of coffee collections is important because they ensure long-term availability of germplasm to guarantee the sustainability of coffee value chain. The conservation of coffee genetic resources is essential to provide the raw materials for breeding and improvement of the crop. Many genetic resources of wild arabica coffee have been collected in the second half of the 20th century by several international collecting missions, including by Food and Agriculture Organization of the United Nations, ORSTOM (now IRD), Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), and IPGRI (now Bioversity International), and are conserved in several national genebanks and at the CATIE International Coffee Collection (CICC) in Turrialba, Costa Rica. Over the past decades, many of the original accessions of the CICC have become threatened due to age, pests and diseases, inadequate management, and waterlogging. There is thus an urgent need to rejuvenate and rationalize the collection to ensure the long-term maintenance of the genetic diversity of the original accessions. Here we present the methodological approach we followed to carry out an in-depth assessment of the status of the coffee collection at CATIE and to prioritize accession-specific actions for the rationalization of the collection. This can be used as a model for other collections to assess and rationalize their own field genebank, with a view to improving their management in the most cost-effective way. The study identified many discrepancies between the number of accessions in the field and genebank records and revealed that 80 accessions have been lost from the collection since 2014 and that approximately 80% of the accessions were threatened and in need of intervention. Furthermore, the in-depth study identified the most diverse and valued accessions for the rationalization of the CICC field genebank and those that are in urgent need of safety duplication.

Keywords: coffee genetic resources, *Coffea arabica*, *ex situ* conservation, field genebank, prioritization

INTRODUCTION

The long-term *ex situ* conservation of coffee genetic resources faces many challenges. It has long been known that seeds of coffee species cannot be conserved under the standard conservation conditions in seed banks for extended period because coffee seeds are only partially tolerant to desiccation and are cold sensitive (Hong and Ellis, 1995). Other techniques for conserving coffee genetic resources *ex situ* have been developed (Dulloo et al., 1998; Engelmann and Dulloo, 2007), including cryopreservation (storage at liquid nitrogen temperature, −196°C) of *Coffea arabica* L. seeds (Dussert et al., 2001) and *in vitro* slow growth and cryopreservation for medium- to long-term conservation of zygotic or somatic embryos, apices, and buds (Dussert et al., 1997). Other options of *ex situ* conservation include pollen storage under vacuum (Walyaro and Van der Vossen, 1977) and DNA storage (Adams and Adams, 1991). All of these methods have their respective advantages and disadvantages. Although *in situ* protection of *Coffea* species and varieties, both in the wild and on farms, is a potentially important conservation approach, it has not received sufficient attention or resources. Consequently, the *ex situ* conservation of coffee genetic resources is mostly done as live plants in field genebanks (Vega et al., 2008; Dulloo et al., 2009). However, this mode of conservation suffers from many drawbacks and is vulnerable to many technical, management, and economic factors, including pest and disease outbreaks, extreme weather conditions, cyclones, fire, suboptimal ecological conditions, land availability, high labor requirements, and high costs (Dulloo et al., 2001, 2009; Bramel et al., 2017). In the long term, the maintenance of field genebanks often becomes a financial burden for institutions, and this may result in poorly maintained collections and the loss of accessions and thus genetic diversity (Dulloo et al., 2009; Bramel et al., 2017).

Coffee field genebanks were established in the second half of the 20th century following major international collecting missions undertaken by the FAO (Fernie et al., 1968), ORSTOM (Guillaumet and Hallé, 1978), CIRAD, the Museum of Natural History of Paris, and IPGRI (Bramel et al., 2017). Countries that harbor important diversity of wild *Coffea* species (Ethiopia, Madagascar, Cote d'Ivoire) and where there is a breeding program have also established their own national coffee field genebanks. Many of the samples collected by the international missions have also been sent to several national field genebank around the world (e.g., India, Ethiopia, Tanzania, Colombia, and Peru) (Fernie et al., 1968). Inventory of coffee field genebanks throughout the world has been undertaken in the past by several authors (Bettencourt and Konopka, 1988; FAO-WIEW Database (cited in Bramwell et al., 2017); Eira et al., 2007; Labouisse et al., 2008; Dulloo et al., 2009; and Phiri, 2013), but the latest inventory reveals a total of more than 21,000 accessions being conserved in field genebanks globally (Bramel et al., 2017).

CATIE International Coffee Collection (CICC), established in 1949 in Turrialba, Costa Rica, is one of the world's largest collections of *C. arabica* and a few diploid coffee species, with 1,960 accessions, which includes samples of the historic collecting missions by FAO, ORSTOM, and IPGRI (Bramel et al., 2017). It is considered as the most important coffee collection in the world in the public domain, given its unique status by virtue of the agreement signed between CATIE and the International Treaty on Plant Genetic Resources for Food and Agriculture under its Article 15 (FAO, 2009). As such, the collection is accessible to users for research, breeding, and training under its facilitated access and benefit sharing arrangements. The collection was identified by the Global Coffee Conservation Strategy, prepared by the Crop Trust and World Coffee Research (Bramel et al., 2017), as essential for the long-term preservation of coffee diversity, one of the so-called "Origin Collections" outside the African continent that would meet the eligibility criteria to receive resources from the Crop Trust's Endowment Fund. In fact, the wild genotypes in the CICC have been used extensively in regional breeding programs in collaboration with different partners to produce highly productive and disease-resistant coffee varieties (Bramel et al., 2017). For example, the hybrid Nemaya was developed from two accessions of *Coffea canephora*, with resistance to nematodes and having a strong root system, being used as a rootstock for regenerating *C. arabica*. Another accession, the Geisha variety, has been shown to have resistance to coffee leaf rust and is widely used in breeding programs (Bramel et al., 2017). This variety was distributed in the 1960s to Boquete area in Panama, where it developed excellent organoleptic characteristics, and became recognized worldwide for its high quality, allowing this variety to reach record figures in international auctions.

The CICC has also been suffering from the loss of some of its accessions, principally due to aging trees, waterlogging, and diseases, principally leaf rust and American Leaf spot (Bramel et al., 2017). Furthermore, given the size of the collection, there has been a lack of adequate operational funds to purchase farm inputs such as fertilizers and pesticides. To ensure long-term sustainable support for management of the historic collection of CICC, the Global Crop Diversity Trust commissioned a study in 2019 to carry out an in-depth assessment of each accession in the CICC with a view to rationalizing the collection and defining strategic conservation actions (Dulloo, 2020a). As part of this study, an accession-by-accession prioritization methodology was developed to help determine a set of the most important accessions in the collection that would be part of the rationalized field collection. The aim of this article is to describe the accession by accession prioritization methodology that has been applied to CICC, to serve as a model that other field genebanks could use to better manage their own collection in a most cost-effective manner.

METHODS

The prioritization process involved carrying out five main steps (**Figure 1**), as follows:

Step 1: Gather, Collate, and Analyze Information on Each Accession

The first step involves gathering and analyzing all management, passport, characterization, and evaluation data for each accession contained in the field genebank that is held on record and

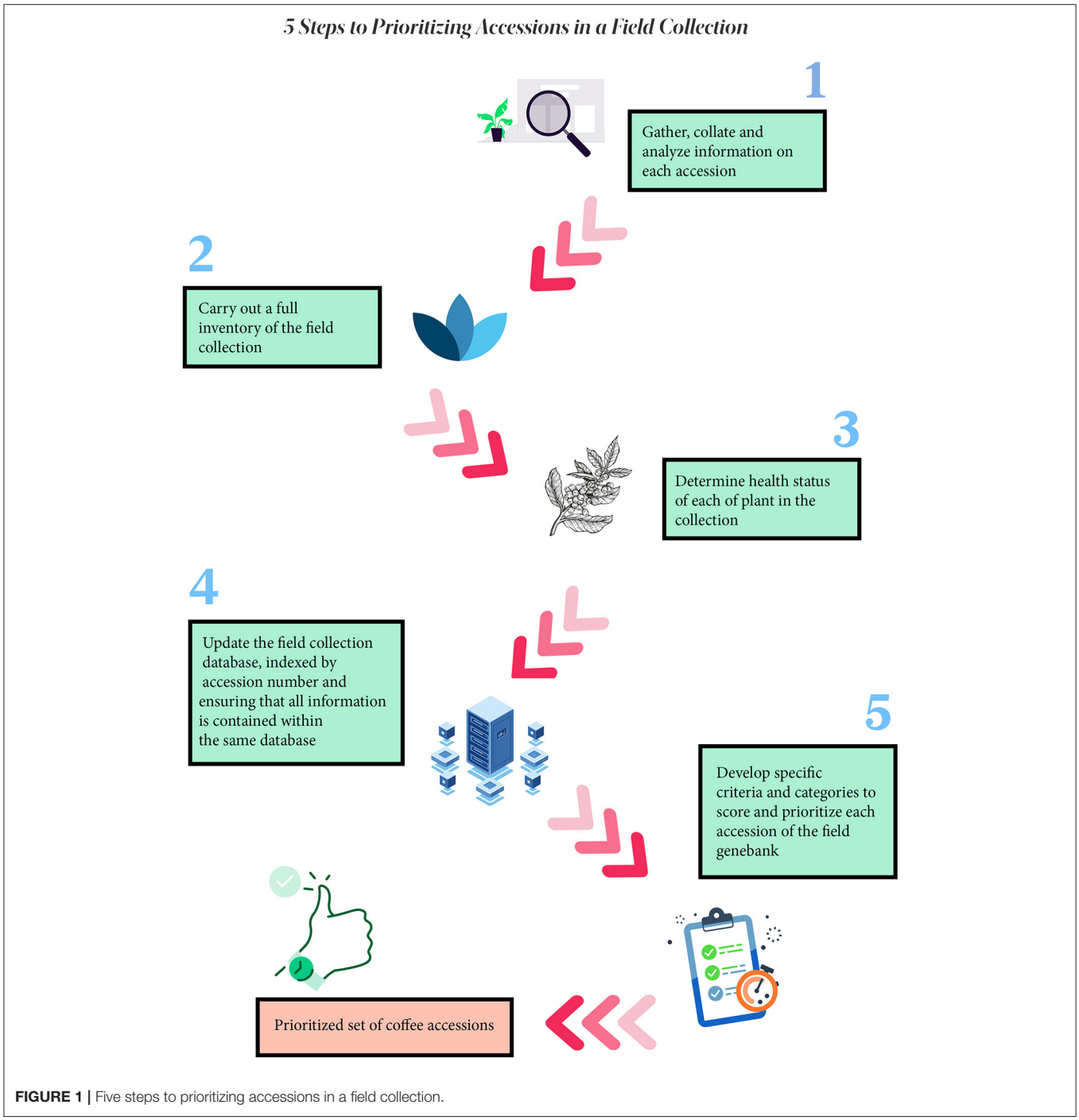

FIGURE 1 | Five steps to prioritizing accessions in a field collection.

identifying any gaps in information on each accession. Often, such information is scattered in different paper and computer files, and it is important that all the information is combined in one database to facilitate analysis. This step also involves carrying out a thorough literature review to gather information about the origins of the accessions and to identify occurrences of the same material in other field genebank, both within and outside the country, to ascertain which accessions may be duplicated elsewhere.

In the CICC, the relevant information was extracted from two principal databases: (i) CATIE's catalog database that contains the passport information (Introduction Book), to verify taxonomy information, varietal name, accession number, specific observations on the accession, its origin, and date of introduction; and (ii) the separate CAFE-BASE database, which also has passport information, but also comprises field inventories and evaluation data. In addition, the results of a 2019 inventory and individual health status assessment of plants

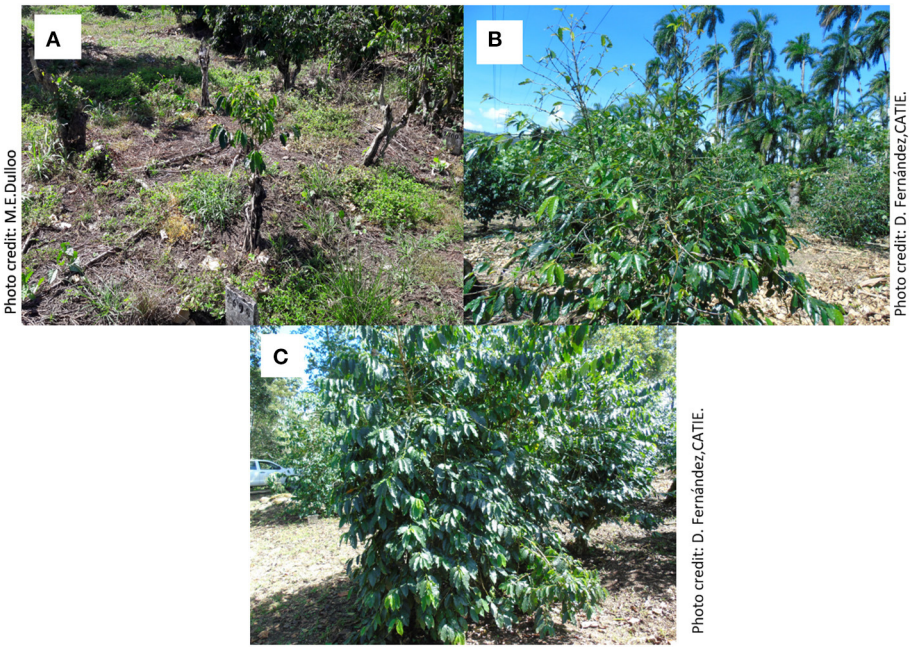

FIGURE 2 | Photographs showing health status of coffee plant in field genebank. **(A)**, Critical; **(B)**, poor; **(C)**, healthy.

of each accession (see below) were held in different files. All the information was brought together in one database to enable a full analysis of the data available for all the accessions of the collection.

Step 2: Carry Out a Full Inventory of the Field Collection

The next step is to undertake a full inventory of the field collection. This involves the physical counting of the number of trees alive for each accession in the field. For each accession extant in the field, the following information should be recorded.

- Accession number.
- Description of the accession.
- The precise location of the accession, depending on how the collection is organized in the field (e.g., section, blocks, lines, etc.).
- Number of plants still alive for the accession: This should be further subdivided as the number of original plants and number of plants that have been regenerated.

A full inventory of the plants within each accession of the CICC was undertaken by the CATIE genebank staff in 2019. Prior inventories of the collection were done in 2008 and 2014.

Step 3: Determine Health Status of Each of Plant in the Collection

It is important that the health status of each plant in the accession be assessed. This involves making a physical observation of the plant in the field and classifying it into one of three categories: critical, poor, and healthy (see criteria and categories below). **Figure 2** illustrates visually the status of coffee plants that are in three health categories. This step is best done at the same time as step 2 above.

Step 4: Update the Field Collection Database, Indexed by Accession Number and Ensuring That All Information Is Contained Within the Same Database

In CICC, all information containing the accession level data on field genebank records in the CATIE catalog, CASE-BASE, inventory files, characterization, and evaluation were analyzed by CATIE information technology specialists who compiled and organized all the information into a single Excel document, using the accession number as the common denominator. This work required reviewing and debugging the information, extracting the data and generating a consolidated field genebank record database, and using unification programming algorithms. In addition, a full bibliographic search for publications containing characterization of coffee from CICC was undertaken from the CATIE's ORTON Library database, as well as from PROMECAFE Network. The extracted information, including 31 titles from ORTON Library and 48 titles from PROMECAFE Network, was compiled in Excel sheets and integrated with the new consolidated field genebank record database.

Step 5: Develop Specific Criteria and Categories to Score and Prioritize Each Accession of the Field Genebank

To prioritize a field genebank collection for appropriate actions, a set of criteria and categories and a scoring mechanism should be developed. It may be necessary to prioritize the criteria and assign

TABLE 1 | Score table for total number of plants and number of original plants in CICC collection.

No of plants within an accession	Score for total number of plants	Score for number of original plants
1	10	5
2	8	4
3-4	6	3
5-6	4	2
>6	2	1

TABLE 2 | Score range for different threat categories.

Score range	Threat status	Color code
0	Extinct	EX
18–20	Critically endangered	CE
15–17	Endangered	EN
10 to <15	Threatened	TR
5 to <10	Vulnerable	VU
1 to <5	Not threatened	NT

different weights to them according to their priority. Each field genebank may develop its own criteria and categories and scoring mechanism depending on the type of crop and the local context.

In the case of CICC, the specific criteria and categories were developed in consultation with the genebank staff and validated by recognized genetic resources experts. Five criteria (type of genetic resources, threat, uniqueness, safety duplication, and use) were identified for prioritizing the accessions of the collection. The "use" criterion could not be used because of the lack of sufficient available information. For each of the criteria, categories were defined and given a score between 1 and 20, depending on their priority. All the criteria have the same maximum score of 20.

Type of Genetic Resources

This criterion identifies the different types of genetic materials that are conserved in the CICC. This criterion is regarded as being the most important and was used to categorize accessions according to whether they were "wild materials" (mostly derived from the historic international collecting missions from Southwestern region of Ethiopia), cultivated varieties, or breeding /experimental materials. The information was derived from the passport data file held in CATIE. Each category was prioritized as follows:

1. Wild materials: 20 points.
2. Cultivated varieties: 10 points.
3. Breeding lines and experimental materials: 5 points.

Threat

The threat criterion quantifies the risk of losing an accession from the collection and is measured by a score combining the current number of plants for each accession and their observed health status. A score is given for the total number of plants for the accession and for the number of original plants (meaning the plants first introduced in the collection) according to the Score Table (**Table 1**). In CATIE, generally an accession is represented by eight plants in the collection (but sometimes more and sometimes less). An accession starts becoming threatened and at risk of loss when it declines below six plants. Thus, the lower the number of plants in the accession, the more threatened it would be, and the higher a high score it received. In addition, we gave extra points for the number of original plants, as we considered these plants as being the "most original plants" and had a higher value compared with others that had been regenerated (**Table 1**).

The health status of the accession was defined by three states, namely, critical, poor, and healthy. Each plant (irrespective of whether it was an original or regenerated plant) was assessed and given a score of 5 points for critical, 3 points for poor, and 1 point for healthy. When there were more than six plants for an accession, only the best six plants were scored. For example, if an accession had nine plants with 4 "healthy," 3 "poor," and 2 "critical," we scored the 4 healthy and 2 poor plants. If an accession had nine plants with 3 "healthy," 2 "poor," and 4 "critical," then we would score 3 "healthy," 2 "poor," and 1 "critical." The mean value for the best six plants was then taken as the health status of the accession as a whole (see **Supplementary Table S1** for more detailed explanation on how to calculate the health status score).

The Total Threat Score was then computed as the sum of the individual scores for total plants, original plants, and health scores for each accession. They were sorted with highest score being more threatened, and we categorized accessions in five groups and named the categories as critically endangered (CE), endangered (EN), threatened (TR), vulnerable (VU), and not threatened (NT), based on the score ranges in **Table 2**. In addition, if an accession was found to have been lost from the collection, it was placed in the extinct category and received a score of 0.

Uniqueness

With this criterion, we were trying to estimate how distinctive an accession was within the collection. Uniqueness was measured by counting the total number of accessions collected from a locality, as per the locality name given in the passport data. In the case of CICC, we were able to apply this criterion only to the wild-derived accession as their precise location is well documented by collecting missions of FAO 1964/65 (Fernie et al., 1968) and ORSTOM 1966 (Guillaumet and Hallé, 1978). If, at given locality, there was only one accession in the collection, it will be more important with regard to this criterion than an accession from a locality from which there are many accessions. **Table 3** gives the score table for uniqueness. To determine the score for the accessions from a locality, we looked at the range of number of accessions per locality, grouped them (**Table 3**), and assigned scores of 0 to 15 from the highest number of accessions to the lowest.

TABLE 3 | Score table for number of locality areas.

Number of accessions from a locality	Score
1	15
2–4	12
5–10	10
11–17	8
23–30	5
>30	0

For each accession, the exact location of collecting has been determined from the passport data file, original reports (Fernie et al., 1968; Guillaumet and Hallé, 1978), and the literature. The number of accessions known to have been collected from each specified site was then compiled. Another consideration was whether the site was from the Southwestern part of Ethiopia, considered as the center of diversity of *C. arabica* (Sylvain, 1955; Charrier and Bethaud, 1985; Bramel et al., 2017). In this study, we considered any accession coming from provinces of Kaffa and Illubabor of Ethiopia as originating from the center of diversity, and it received an additional 5 points. The maximum score for this criterion was 20.

Note that an accession can also be unique if it is only present in the CATIE collection and in no other collection. However, this could not be accounted for under this criterion because of insufficient information from other coffee collections around the world (see *Safety duplication*).

Safety Duplication

This criterion refers to whether an accession held in the collection is safely duplicated in another genebank, within or outside the country, for which ideally there should be an official signed agreement between the two institutions. However, in practice, if an accession is known based on a reputable source to be found in another collection, it should be counted as being duplicated, although its security may not be guaranteed. If an accession is not known to be safety duplicated, then it is considered as highest priority and receives a maximum score of 20. Depending on the number of collections in which an accession is found, it receives successively fewer points. Thus, the scoring for safety duplication is as follows:

a. No evidence of safety duplication (20 points).
b. Safety duplicated in 1 collection (15 points).
c. Safety duplicated in 2 collections (10 points).
d. Safety duplicated in 3 or more collections (5 points).

In the case of CICC, data on safety duplication were obtained from three other coffee collections, namely, US Department of Agriculture (USDA), Fort Collins, CO, USA; the Nica-France Foundation farm at La Cumplida, Nicaragua; and the Institut de Recherche pour le Développement, France (IRD) collection at La Reunion (personal communication with Stephanie Greene, Melanie Bordeaux, and Thierry Joet, respectively).

RESULTS

Composition of the CICC

The 2019 inventory of the CICC revealed a total of 1,975 accessions in the field, organized in 10 sections, labeled A to H, Musas and Citricos, in CATIE's campus. However, when compared with records of the passport data and introduction files, there was a disparity among them, summarized as follows:

- 172 accessions of 2019 inventory without passport data.
- 18 of 2019 inventory without passport data and not found in introduction book.
- 111 accessions on records with passport data, but not in the field.
- 130 accessions recorded in introduction book, but not in field.
- 47 accessions found in the field (2019 inventory) with no accession number (these were hybrid varieties from CATIE's breeding program).

After accounting for these disparities, the total number of accessions remaining in the CICC as of December 2019 was 1,895 accessions.

The collection was made up principally of arabica coffee, with 91.93% (including introgressed and other interspecific hybrids), followed by *C. canephora* Pierre ex Froehner (4.06%) and *Coffea liberica* Bul ex Hiern (1.27%). Other species with very low percentages (<1% each) included *Coffea sessiliflora* Bridson (14 accessions), *Coffea brevipes* Hiern (7 accessions), *Coffea pseudozanguebariae* Bridson (11 accessions), *Coffea eugenioides* S. Moore (6 accessions), *Coffea racemosa* Lour. (3 accessions), *Coffea salvatrix* Swynn. & Philipson (2 accessions), and *Coffea congensis* A. Froehner (1 accession). There were eight accessions for which the species is unidentified (**Figure 3A**).

The CICC may be broken down as follows with regard to the type of genetic resource, in order of priority (**Figure 3B**):

1. Wild materials (661 accessions)
2. Cultivated varieties (443 accessions)
3. Breeding lines and experimental materials (784 accessions).
4. Unknowns (blanks) (7 accessions).

The wild material included mostly accessions collected during the FAO collecting mission 64/65 in Ethiopia (E series) (424 accessions [64%]) and the ORSTOM collecting mission (ET series) (90 accessions). It also included materials collected by IPGRI in Yemen (nine accessions). In addition, some clonal materials (*in vitro* plants) were received form ORSTOM (97 accessions). The remaining accessions were mostly diploid species that came from other sources, including CIRAD, and countries from South America.

It must be noted that many CICC accessions arising from the ORSTOM mission (identified by their ET codes) were introduced in CICC from three different sources (**Supplementary Table S2**). Consequently, there were three sets of accessions for the same material, but with different CICC accession numbers, totaling 155 accessions. Fifty-two accessions were received from Institut de Recherches du Cafe et du Cacao (IRCC), Paris (with IRCC numbers) in 1985 (T.16689 to T.16741); 45 accessions were received from Institut de Recherches du Cafe et du Cacao,

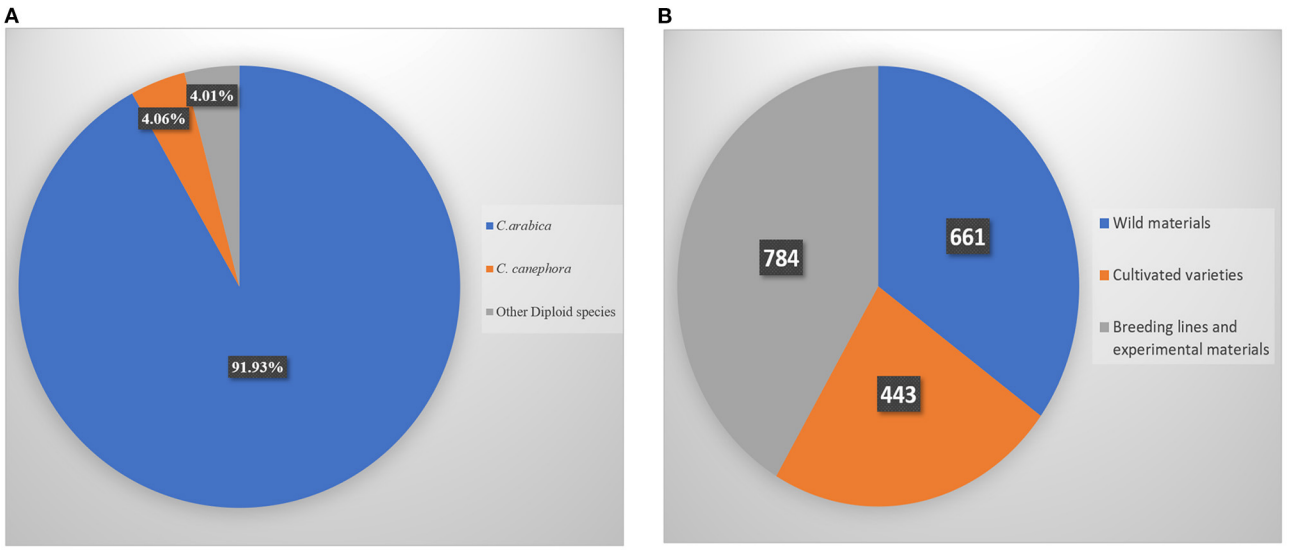

FIGURE 3 | (A) Species composition of CATIE International Coffee Collection; other diploid species include *C. sessiliflora*, *C. pseudozanguebariae*, *C. brevipes*, *C. eugenioides*, *C. racemosa*, *C, salvatrix*, *C. congensis*, *C. spp*. **(B)** Number of accessions of types of coffee genetic resources in CATIE International Coffee Collection.

TABLE 4 | Number of accessions in threat categories [Brackets under wild total represent the effective numbers of accessions, after accounting for duplications of ORSTOM accessions (ET codes)].

Threat categories	Code	Score range	Wild *C. arabica*	Wild diploids	Wild total	Cultivated varieties *C. arabica*	Combined
Extinct	EX		6	23	28	19	47 (+33 other types)
Critically endangered	CE	18–20	13	9	22 (14)	27	49
Endangered	EN	15–17	45	17	62 (38)	41	103
Threatened	TR	10–<15	166	36	202 (155)	166	368
Vulnerable	VU	5–<10	215	19	234 (212)	145	379
Total threatened			**439**	**81**	**520**	**379**	**899**
Not threatened	NT	1–<5	140	1	141 (120)	64	205
TOTAL			**579**	**82**	**661**	**443**	**1104**
% threatened					**78.6%**	**85.6%**	**81.4%**

Nkolbisson, Cameroun with code cX (number of Bouharmont) or L series number, received in 1986 (T.17173 to T.17242) (Dulloo, 2020a). It was uncertain from where these materials were derived. It could have been introduced from the ORSTOM missions in West and East Africa in 1970s and 1980s (Charrier and Bethaud, 1985). Furthermore, 58 accessions were received as clones introduced and multiplied *in vitro* from ORSTOM, Montpellier, in 1995 (T.21259 to T.21316). Thus, the same ORSTOM mission population (ET code) had been introduced and assigned a different CATIE accession number (T series). For example, accessions T.16689, T.17173, and T.21259 all represented the same ORTSOM mission population ET-01 and were introduced from Paris, Cameroon, and as *in vitro* material from Montpellier, respectively.

In this study, we focused on the wild material and cultivated arabica varieties as being the top priority from a conservation perspective; for each of these two groups, the accessions were ranked using the criteria and categories for which data were available.

Lost Accessions

According to the 2019 inventory, 80 accessions from the collection were lost (**Table 4**). Fifty-six accessions were of *C. arabica*, 23 accessions were of diploid species, and one unknown species (T.04466). Among diploids, the largest losses were those of *C. racemosa* (six accessions), *C. pseudozanguebariae* (five accessions), and *C. eugenioides* (three accessions); *C. canephora*, *C. brevipes*, and *C. sessiliflora* had each lost two accessions, whereas the accessions *C. congensis* (T.04098), *C. liberica* (T.02536), and *Coffea stenophylla* (T.03416) were also lost.

Of the 80 lost accessions, 28 accessions were of wild materials, 19 were cultivated varieties, and 30 were of breeding lines or experimental material and three unknown materials (T04466, T05056, T055058) (**Table 4**). Only two accessions (T.04538-E292 and T.04819-E419) of the FAO collecting mission 1964/65 had been lost from the historic collection. Four accessions of the ORSTOM collection were also lost (T.17224 [ET-39], T21284 [ET24], T21291 [ET029B], and T.21295 [ET-33B]). The last three accessions were *in vitro* materials received from France.

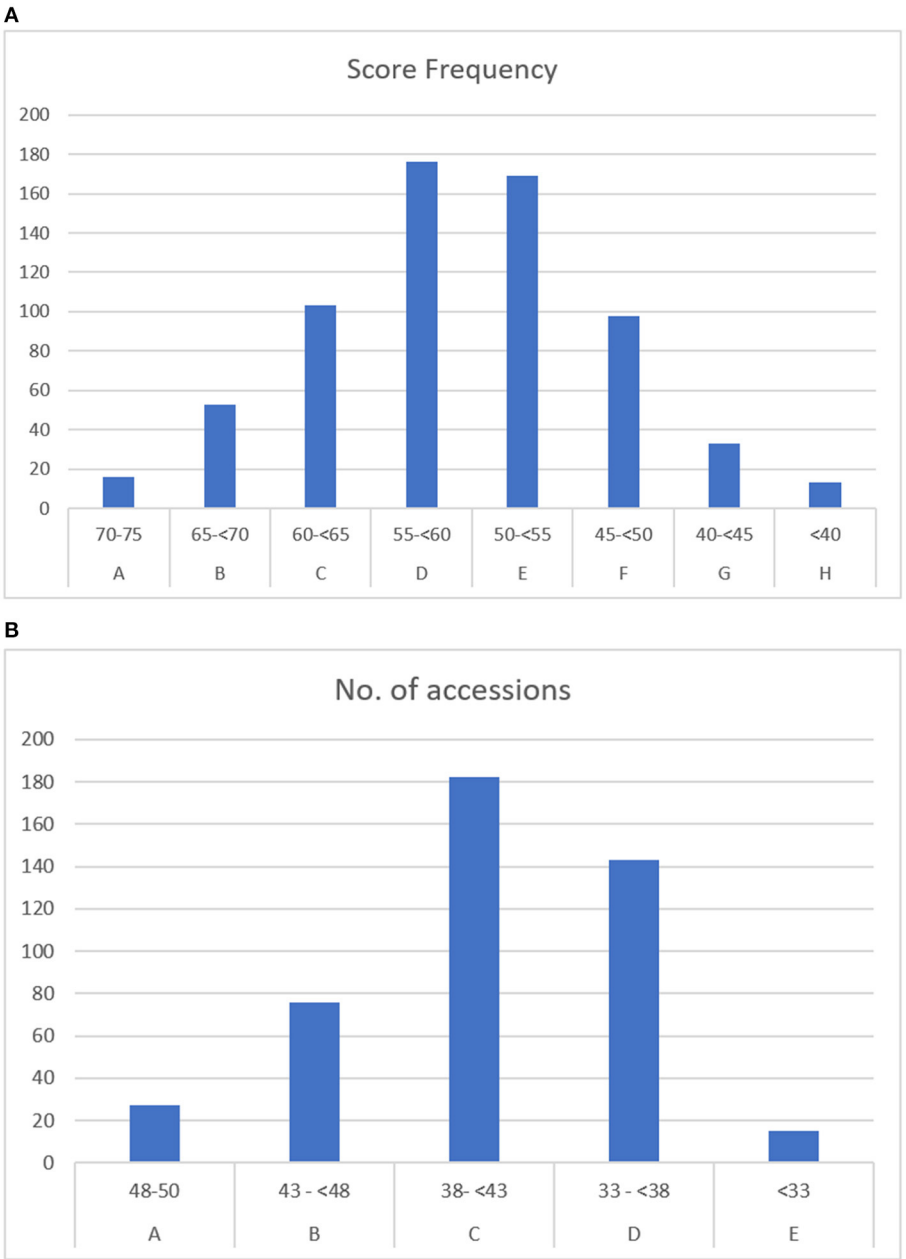

FIGURE 4 | (A) Overall score frequency of wild accessions in priority groups A to H. **(B)** Overall score frequency of cultivated accessions in priority groups A to E.

Status of Wild Material

The overall prioritized accessions of the so-called wild materials were divided into eight groups according to score range (**Figure 4A**). The highest-priority accessions were those belonging to groups A to C (having a score of ≥60), giving a total of 172 accessions of wild materials.

Threat Status of Wild Materials

Besides the 28 accessions lost from the collection, there were 22 accessions of the wild materials considered as critically endangered (**Table 4**), of which five were *C. arabica* accessions collected from the FAO collection mission 1964/65 collection, eight are accessions of *C. arabica* from the ORSTOM mission, and nine were diploid accessions received as *in vitro* clones from ORSTOM Montpellier.

The most threatened accessions are the accession T.04738 (*C. arabica*, E215) and the two diploid accessions T21320 (*C. pseudozanguebariae*) and T21359 (*C. racemosa*), which received the maximum score of 20. They are all represented by a single remaining plant in the collection, and their health status is critical. Accession T.04848 is the next most threatened accession, with 19 points. In addition, there are three wild

TABLE 5 | Accessions of ET codes that are critically endangered.

ET code	Accessions critically endangered	Comments	ET code combined treat status
ET-01	T.21259	Represented by two other accessions of T.17173 and T.16689, that have in combination a total of 18 plants of which 11 plants are in healthy state	NT
ET-20	T.21279 and T.21280	Also represented in the collection under 3 other accessions numbers – T.17236, T.17205, T.17206. Combined, there are 16 individual plants and 9 are healthy.	NT
ET-32B	T.21292	Also represented in the collection under 3 other accessions numbers – T.17236, T.17205, T.17206. Combined, there are 16 individual plants and 9 are healthy.	NT
ET-38	T.21301	Also represented in the collection by another accession T 17223, which has 9 plants, of which 6 plants are healthy	NT
ET-41	T.21303	Represented in the collection by another accession (T 16725) which has 11 plants, of which 8 plants are healthy. It had another accession, T.17201, that have been lost	NT
ET-44	T.21304	Represented in the collection by another accession, T 16728, which has 6 plants, of which 4 plants are healthy	NT
ET-52	T.21308	Represented in the collection by another accession, T 16728, which combined have 6 plants, of which 4 plants are healthy.	TR

accessions (T.04704, T.04768, and T.04868) from the FAO mission originating from the center of diversity in Kaffa and Illubabor that are critically endangered.

The multiple introductions of the same ORSTOM material posed some challenges in the assessment of the threats of these CICC accessions. A separate analysis of the ET series codes was carried out (Dulloo, 2020a), and a combined threat score was calculated for the same ET code accessions (**Supplementary Table S3**). The results here showed that none of the ET codes were critically endangered, although eight CATIE accessions from ORSTOM mission were classified as critically endangered (**Table 5**). As there were other CICC accessions representing the same ORSTOM mission population, the combined assessment gave a "not threatened" result, as together they would have a greater number of healthy plants, thus making them not critically endangered. For example, CICC accession T21259-ET-01 was scored as critically engendered, but the same population (ET-01) was represented by two other accessions (T.17173 and T.16689), which have in combination a total of 18 plants, of which 11 plants were in healthy state. When assessed in combination, the ORSTOM mission population ET-01 was considered as not threatened. Thus, taking this into account, only 14 accessions in CICC were considered as "critically endangered" (**Table 4**).

There were 62 accessions that scored between 15 and 17 points and were considered as "endangered." Nearly 80% of them (49 accessions) were from the ORSTOM mission, and a significant number had been received as *in vitro* clones. Seven populations collected by the ORSTOM mission were represented by a single accession, but the remaining 26 populations had more than one accession, and when these were combined, they were not endangered. Two accessions (T.17181 [ET-11C] and T.17241 [ET-35D]) were represented by several other accessions in the collection, but they were the original germplasm from Cameroon that should be considered as priority to conserve. They were represented by only one or two plants in the collection and were thus endangered. Thus, there were 38 accessions that were effectively "endangered," of which 20 were *C. arabica*.

Regarding the "threatened" accessions, there were 202 accessions, with 50% coming from the FAO mission. As with other categories, many of the unique ORSTOM mission populations were represented by multiple CICC accessions. Consequently, of the 202 accessions, only 155 were effectively threatened, of which 119 were *C. arabica* accessions.

The great majority of "vulnerable" accessions (189 of 234) were *C. arabica* from the FAO mission. Four populations of the ORSTOM mission were represented by a single accession that is vulnerable. In addition, there were several combinations of accessions with a single ET code that together gave a vulnerable threat score. Consequently, of 234 accessions, there were 212 accessions that were effectively vulnerable (with 196 of *C. arabica* accessions). In addition, there were 10 combined sets of accessions of ET series codes that were considered as "vulnerable."

The remaining 141 accessions were considered as "not threatened," with a total score of less than 5 (**Table 4**). They included 115 *C. arabica* accessions from the FAO mission, 25 *C. arabica* accessions of the ORSTOM mission, and only one accession (T21329) of *C. canephora* (*in vitro* clone from ORSTOM). However, among the 25 CATIE accessions of the ORSTOM mission, there were four ORSTOM mission populations ET-03, ET-09, ET-26, and ET42 that were represented by a single CATIE accession, namely, T.16691, T.16697, T.16713, and T.16726, respectively, and were not threatened (**Supplementary Table 3** [Annex 8-ET codes]). For the remaining 21 CATIE accessions, there were other accessions that represented the same ORSTOM mission population, which belonged to the threatened category. Consequently, in total, there were 120 accessions that were effectively "not threatened."

Uniqueness

Table 6 shows the number of accessions that belong to the different "uniqueness" categories, within and outside the center of diversity (see *Methods* section). Unfortunately, we do not have the full passport data (especially the site information) on all the accessions in the collection. This limits the kind of analysis

that we can do, and overall, the uniqueness criterion is difficult to apply to all accessions in the CICC. It was only possible to do this analysis for the accessions for which information on locality names was available, which included mostly the historic collection missions of FAO (Fernie et al., 1968), IPGRI, and ORSTOM (Guillaumet and Hallé, 1978).

The most unique accessions from the FAO and ORSTOM missions were those that came from only a single locality within the center of diversity. There were five accessions that satisfied these criteria and scored the maximum score of 20 points (**Table 7**). These accessions were not highly endangered and were either VU or TR. There was also a set of 20 accessions (**Table 8**) that came from only one locality, but not within the center of diversity.

Status of Cultivated Material

The 443 accessions of the cultivated materials were ranked using the criteria of germplasm type, threat score, and safety duplication. The accessions were grouped into five groups (A–E) (**Figure 4B**), with the highest priority the accessions in groups A and B (scoring ≥43 points), which contained 103 accessions, 27 from group A and 76 from group B. It is interesting to note that all the accessions from group A were introduced into the collection in the 1950s and 1960s. None of the accessions in groups A and B were safety duplicated. There were six accessions that were duplicated in group D, and the rest of the duplicated materials were from group E. This means that none of the higher-priority groups were duplicated, and only a few of the least important accessions were duplicated.

TABLE 6 | Number of accessions within and outside center of diversity.

Uniqueness categories	Number of accessions in center of diversity (Provinces of Kaffa and Illubabor)	Number of accessions outside center of diversity	Total
1	5	20	25
2–4	24	17	41
5–10	60	23	83
11–17	88	37	125
23–30	99	32	131
>30	99	69	168
Subtotal	**374**	**198**	**572**
Blanks			88
TOTAL			**661**

Threat Status of "Cultivated Materials"

Table 4 gives the summary of the number of accessions of cultivated materials belonging to the five threat groups. There were 19 accessions of cultivated varieties that had been lost from the collection as per the 2019 inventory. Twenty-seven accessions of cultivated varieties were critically endangered and were represented by a single plant in the collection. Among them, there were seven accessions for which the state of health of the single plant left is critical. These needed to be salvaged as a matter of urgency. The other 20 accessions were also in a poor state and needed to be salvaged as soon as possible. These accessions were also not duplicated elsewhere (i.e., in USDA and La Cumplida farm).

There were 41 "endangered" accessions, of which 11 accessions were represented by only a single plant, but they were all healthy, and the rest were represented by two plants only. Among the latter, there were six accessions (T.02544, T.03685, T.04295, T.04310, T17557, T.02699, and T.17931) whose health

TABLE 8 | Accessions from one locality outside center of diversity (*C. arabica*).

CICC accession number	Identification	Country of introduction	Locality name
T.04472	E-007	Ethiopia	Combulchia
T.04500	E-036	Ethiopia	Welkitte
T.04612	E-089	Ethiopia	Gera- Jimma
T.04622	E-124	Ethiopia	Geisha
T.04623	E-125	Ethiopia	Tui
T.04624	E-126	Ethiopia	Gorei -Geisha
T.04758	E-237	Ethiopia	Yirgalem
T.04759	E-238	Ethiopia	Aleta Wondo
T.04892	E-524	Ethiopia	Omonadda
T.04945	E-579	Ethiopia	Faghena Erythrea
T.04946	E-001	Ethiopia	Finote Selam
T.04950	E-012	Ethiopia	Harar city
T.04952	E-020	Ethiopia	Dilla
T.21231	PDRY-01	France	Hashsh
T.21234	PDRY-05	France	Dinakheb
T.21236	PDRY-07	France	Hanaka
T.21237	PDRY-09	France	Baynassyal
T.21239	PDRY-014	France	El-Kor
T.21240	PDRY-15	France	Diraa
T.21242	PDRY-22	France	Hewle

TABLE 7 | Most unique accessions in the FAO and ORSTOM collections (*C. arabica*).

CICC accession number	Identification	Country of introduction	Locality name	Center of diversity	Treat status
T.04775	E-272	Ethiopia	Kolu	kaffa	VU
T.04780	E-277	Ethiopia	Komba Agaro	kaffa	TR
T.04781	E-287	Etiopia	Shebe	kaffa	VU
T.04924	E-555	Ethiopia	Kaffa	kaffa	TR
T.17231	ET-45	Cameroon	Filoa Hotsprings	kaffa	TR

status was all "critical," except accessions T.04310 and T17931, which had one plant as "critical" and one plant are "poor." These accessions were thus of highest priority in this category considering health status.

One hundred sixty-six accessions of cultivated varieties were threatened, with three accessions represented by a single individual (T17545, T.17548, and T.17549) and were all in poor health. Twenty-seven accessions were represented by two plants, with 10 accessions healthy and the remaining accessions with one plant either healthy or poor. Furthermore, there were 66 accessions with three plants. Only nine accessions of these were healthy. It should be noted that accession T.04308 has only three plants, all of which were in a critical condition and were among the top two most threatened accessions. There were 63 accessions that were also represented by four plants, four accessions by five plants, and three accessions by six plants.

Among the 145 vulnerable accessions, the number of plants per accession varied from 3 to 19. There were two accessions (T.17541 and T.05038) with no original plants and were represented by three regenerated plants. There were eight accessions that were having a high health status score (3 points), and the rest of the accessions had fewer than 3 points and were doing fairly well. These accessions with high numbers of plants (>8) can be rationalized to reduce the size of the collection.

There were 64 accessions that were considered as "not threatened" and were all doing very well. The outlier was the variety Geisha (T.02722), which was represented by no fewer than 276 plants in the collection. However, the majority of the plants were used for producing seeds for distribution and were not strictly part of the collection. The rest of the accessions had 7 to 19 plants. These accessions with very high numbers of plants (>8) can be rationalized to reduce the size of the collection. It was suggested that only eight plants of Geisha (T.02722) be kept in the rationalized long-term collection and the rest kept in a working collection.

Safety Duplication

The CICC has 221 accessions, representing 11.7% of the total, which are considered as being safely duplicated in three institutions outside Costa Rica, namely, USDA, Nica-France Foundation, and IRD-La Reunion. Of these, only USDA has a formal agreement with CATIE for safety duplication under black-box conditions in cryopreservation. The safety duplicates in the Nica-France Foundation and IRD-La Reunion are both held in field genebanks, but IRD also holds its collection in cryopreservation. Besides these, there is a high probability that some of historic collections are also being conserved in the genebanks in Ethiopia, Tanzania, India, Peru, and elsewhere (Brazil, Colombia), as materials from the historic collections are known to have been sent to these places (Fernie et al., 1968). However, we have no information to date about which accessions are still extant in these national collections. It is recommended that these collections should also be studied using the methodology described in this article to determine which accessions they share in common.

DISCUSSION

Prioritization Methodology

Coffee field genebanks have often been criticized as being highly vulnerable (Vega et al., 2008; Dulloo et al., 2009; Bramel et al., 2017). Most field genebanks in the world suffer from the vagaries of changing climate and weather, inappropriate field conditions, pest and disease outbreaks, fire, and aging plants (FAO, 2013; Alemayehu and Merga, 2017; Bramel et al., 2017). In addition, collections keep growing with time as new accessions are added, and this makes the collection more difficult and expensive to maintain, with the results that the collection is not properly curated, labels are lost from the field, and records are not well kept. These technical, management, and economic constraints severely impact the sustainability of field genebanks, in general, and coffee field genebanks, in particular. In this article, we describe the application of an accession-by-accession methodology to effectively monitor, prioritize, and rationalize the field coffee genebank maintained by CATIE under Article 15 of the Plant Treaty. This methodology can be used by other field genebank curators to assess the status of their collection and ensure that they are properly managed in a cost-effective manner and at high international standards, as recommended by FAO (2013).

The five criteria and categories proposed (type of genetic resources, threat, uniqueness, safety duplication, and use) are key to the prioritization process. However, their successful application is highly dependent on the availability of the relevant information. In this study, the "use" criterion was initially regarded as a very important criterion because it documents the value to the accession and promotes its use, which should be the ultimate goal for maintaining the collection. The "use" criterion should consider the different traits that are important for coffee industry and include resistance to rust and nematodes, cupping quality, biochemical profile (caffeine, chlorogenic acid, sucrose content), resilience to climatic factors, yield, vigor, male sterility, and level of heterozygosity. However, the CICC has not been evaluated for all these traits, and only partial information is available, which makes this criterion impossible to use for prioritizing the entire collection. For example, approximately 50% of the collection has been evaluated for vigor, whereas only 25 accessions have been tested for their organoleptic characteristics. A limited number of accessions have been evaluated, and the characteristics evaluated for some accessions include tolerance to rust, nematodes, vigor, physical characteristics of the grain, and organoleptic qualities. The World Coffee Research has also characterized 847 wild accessions using molecular markers (Klein et al., 2016). Currently, the characterization data of 7 traits for only 34 accessions are made available and are uploaded on the Genesys portal https://www.genesys-pgr.org/; however, the full passport data set of the complete CATIE coffee collection can be found on the portal.

Among the remaining four criteria, the type of genetic resources, that is, whether they were of wild origin, cultivated varieties, or resulting from experimental and breeding activities, was considered as the most important criterion given that it has been demonstrated that wild types are genetically more diverse

compared with cultivated arabica varieties (Lashermes et al., 1996; Scalabrin et al., 2020). However, it is important to clarify that among the so-called "wild materials" accessions in CICC, very few were truly from the wild (Fernie et al., 1968). This was confirmed by a molecular genetic diversity study of the historic collection in CICC, indicating that less than 1.5% of the so-called wild accessions were actually derived from the wild and small farms (Klein et al., 2016).

With regard to the uniqueness criterion, it could only be applied to the so-called wild materials, where the names of the sites from where the seeds were collected, during the FAO and ORSTOM collecting missions, were available (Fernie et al., 1968). This criterion is also considered as being important as it informs us about the origin of the accessions and whether they came from the same or different sites and provides an indication of the genetic distinctiveness of the subpopulations from which they originate. Geographic distribution is often considered as a good proxy for genetic diversity (Pelletier and Carstens, 2018). Thus, the extent of accessions from different locations can be a good indication of the breadth of genetic diversity conserved in the field collection. Often, field genebanks contain a disproportionate number of accessions from the same subpopulation, and knowledge of this can inform how to rationalize the collection to maximize cost-effectiveness in genetic resources conservation, as well as identify gaps in collections. Where feasible, the use of molecular markers would help significantly in characterizing the genetic distinctiveness of the accessions and be used to score this criterion.

In the threat criterion, the category "number of original plants" was considered important because they are the most original plants that were planted and not regenerated and thus was given extra points. It is true that genetically this should not make a difference in terms of genetic diversity, if clonally propagated. However, the fact that the original trees of an accession persisted in the collection can be an indication that they are more adapted to the field conditions than those accessions that have lost their original plants and also to be less likely for errors to be made.

The multiple introductions of the same genetic resources in a collection can make the genebank management at the accession level become complicated, as illustrated by ORSTOM missing introductions in this study. It is important that new introductions be properly screened to see if similar genetic materials do not exist anymore and that records are properly cross referenced. In the case of ORSTOM mission populations, the same materials were introduced from different sources, but in different forms, as plantlets and *in vitro* materials. It was interesting to note that among the accessions that have been introduced to the CICC as *in vitro* clonal materials from ORSTOM, a relatively high proportion are doing poorly compared with the original accessions, and eight accessions (T.21259 to T.21308) were critically endangered (Dulloo, 2020a). This may suggest that plants derived from *in vitro* propagated materials may have a shorter longevity in the field compared with those propagated by cuttings or seeds. However, more research is required to verify this.

Composition of the Accessions Conserved at CICC

The Global Strategy on Coffee Genetic Resources (Bramel et al., 2017) reported that CATIE's accessions from the historic collecting missions of 1964/1965 in Ethiopia by FAO (Fernie et al., 1968) and of 1966 by ORSTOM (now IRD) (Guillaumet and Hallé, 1978) constitute approximately 40% of the conserved accessions. The material from the IPGRI collecting expedition in Yemen (Eskes, 1989) is represented by only a few (17) accessions. However, the present study has shown that the FAO collection makes up of 22% of the collection (424 accessions), ORSTOM 8% (145 accessions), and only nine accessions (0.5%) come from the Yemen expedition. This difference may be partly due to the fact that the collection may be losing accessions; the study showed that 80 accessions have actually been lost since. But it also may be due to new materials arriving, mainly as breeding lines and experimental materials. The collection lost a disproportionate number of diploid species (23 accessions) such as *C. racemosa*, *C. pseudozanguebariae*, and *C. eugenioides*, *C. canephora*, *C. brevipes*, and *C. sessiliflora*, compared with *C. arabica* (56 accessions), which make up more than 90% of the collection. This is probably due to the low adaptive potential of these diploid wild species to grow under the environmental conditions existing at CICC. They generally exhibit narrow climatic envelopes with restricted habitat (niche) specificity and are mostly forest dwelling.

Rationalization of the CICC Collection

The application of this prioritization methodology to CICC allowed a set of recommendations for the rationalization of collection at CICC and to define cost strategic conservation actions, including re-establishment and safety duplication of the CICC (Dulloo, 2020b). It is recommended that the so-called wild collected accessions (661 accessions) and cultivated varieties (443 accessions), a total of 1,104 accessions, are included in the rationalized collection, as they represent the most diverse and high-value material in the collection and that the breeding lines and experimental materials including all hybrid materials be moved to separate working collection. This recommendation would allow for a more manageable cost-effective rationalized collection to be established. Furthermore, it was recommended that the number of individual plants in each accession be brought down to six individual plants, keeping the original plants as far as possible. In cases where the numbers are fewer than 6, they should be multiplied urgently to bring back the numbers to six per accession.

The in-depth study (Dulloo, 2020a) also showed that there are 899 accessions (81.4%) considered as being threatened. There were 22 accessions of wild collected genetic material and 27 accessions of cultivated materials that are critically endangered, 49 accessions in total. These are accessions that are down to the last individual that has a critical or poor health status. Immediate action needs to be taken to propagate them by the best available technique to ensure their survival and be replanted in a new location of the rationalized collection (Dulloo, 2020b). There are also 62 accessions from wild collected materials and 41 accessions

cultivated varieties that are endangered, 103 accessions in total. These are accessions that are down to one or two plants and have poor or healthy plants and also need to be rescued urgently.

The safety duplication of CICC was also considered as a high priority, and the study report (Dulloo, 2020b) recommended that priority for safety duplication be given to the historic collection of *C. arabica* belonging to the most threatened groups and that diploid species should be safety duplicated as live plants in another collection site suitable for these species. A total of 403 accessions were identified for urgent safety duplication in cryopreservation.

CONCLUSION

The in-depth study of the CICC allowed its management to carry out a full inventory of the field collection in December 2019, including an assessment of the health status of each tree across all surviving accessions, and to reassemble all its field genebank records into one database system, which henceforth will greatly facilitate the monitoring of the coffee field genebank. It allowed the management to reconcile its records on file with what is actually conserved in the field. Furthermore, the study also allowed CATIE to develop a full rationalization plan for its collection. A new site has been identified, and work has started for the multiplication of 1,104 priority accessions, which will be part of the new collection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MD, WS, DD, and LG conceived the work. MD designed the work. CA acquired the material. MD analyzed and interpreted the data. MD drafted the manuscript which was reviewed by LG, CA, and DD. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.777415/full#supplementary-material

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Smallholder Coffee in the Global Economy—A Framework to Explore Transformation Alternatives of Traditional Agroforestry for Greater Economic, Ecological, and Livelihood Viability

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Sixty percent of global coffee is produced from farms of <5 ha. Studies show that returns from such farms do not generate a living income for producers or workers threatening supplies. Smallholders use agroforestry to reduce coffee production costs, diversify income and address livelihood needs. We undertook a three-phase analysis to test the following hypothesis. Current coffee agroforestry must shift from a low labor, low risk-stable return, slowly-changing matrix to more active management of species and stem turnover in system renovation cycles targeted to sustaining, reorienting and intensifying ecosystem-based benefits to coffee production, diversified income and household food. First, we conducted a document survey of current traditional tree diversity, research trends, and market drivers for more benefits-oriented agroforestry. Second, we proposed a framework for multiple benefits quantification converting tree use characteristics and density into five categories of benefits, each with sub-categories which we tested using previously collected data of stem density by species from coffee agroforestry in northern Nicaragua. Third, we modeled radiation in mixed canopy scenarios using the program SExI- FS based on modifications of species and density to target food and income diversification and tested our framework by quantifying benefits. We found that smallholder coffee faces farms decreasing coffee margins, labor scarcity, new pests and climate variability best addressed with targeted and adaptive shifts in coffee varieties and associated trees. Increasing data demands from certification and regulations provide a basis more data-driven coffee farm management. Our data bases of stem density by species of established agroforestry systems were sufficient to identify gaps in food and income benefits which were addressed in the scenarios thereby verifying the hypothesis. The benefits ranking both of current systems and three scenarios also provided insights into data collection specifications for a more rigorous academic test of the hypothesis and data-driven grower strategies for agroforestry transformation.

Keywords: coffee agroforestry, agroecology, agroecosystem design, ecological intensification, ecosystem services

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INTRODUCTION—CAN AGROFORESTRY TRANSFORMATION ENSURE SMALLHOLDER COFFEE PRODUCTION?

Smallholders with <5 hs supply 60% of the global coffee market (Panhuysen and Pierrot, 2020 referencing Enveritas, 2018) with farms between 5 and 50 ha producing another 19% of world supply (https://carto.com/blog/enveritas-coffee-poverty-visualization/). Coffee price increases in the past year appear to offer some respite, offset by increases in input and transport costs. However, numerous studies summarized by Muñoz-Rodríguez et al. (2019) and Sachs et al. (2019) suggest that over the past decade costs of production have been greater than prices received. The returns provided by smallholder coffee are below a living income both for producers and workers even without taking into account external environmental costs of increasing input use calculated by the True Price method (https://trueprice.org/monetisation-factors-for-true-pricing/). Unfavorable returns may become a threat to global supplies as growers shift to other crops and the sons and daughters of growers and workers seek more promising livelihoods (Giller et al., 2021). Of the estimated 12.5 million coffee-producing small farms, 44% earn below the poverty line (https://carto.com/blog/enveritas-coffee-poverty-visualization/), and a far greater percent do not earn a living income, an income which provides a nutritious diet, decent housing, adequate income to cover education, clothing, health and transport and a reserve for unexpected expenses (https://www.living-income.com/the-concept). A prosperous income level which provides opportunities to progress in life with future generations continuing to produce coffee was proposed by International Coffee Organization (2020) as a goal for the sector.

Fortunately for coffee consumers, smallholder coffee is characterized by inelastic supply (Sachs et al., 2019). Small coffee growers have sunk costs in plantation establishment and on-farm infrastructure. Alternative non-perishable agricultural enterprises are not abundant for remote hilly upper watershed lands and may offer even lower household income from small land areas. On the short term most smallholder coffee producers continue to produce coffee even when prices are low. However, the conditions behind the current inelastic supply may not be enough to ensure the next generation of smallholder coffee growers.

An additional factor favoring the inelastic supply is the diversified agroforestry production system developed by smallholder coffee growers throughout the tropics which harness trees and other associated crops to reduce costs and input needs for coffee and address multiple dimensions of the living income from within the coffee field—food, fuel, housing materials, secondary income, and emergency cash. We propose to focus on these benefits or ecosystem services with a concrete use value locally to smallholder coffee households. Diversified multi-strata coffee is a variant of a very common farmer-originated cropping strategy based on mixed species plantings with complementary plant architecture and crop cycles practiced with the beginnings of agriculture (Rindos, 1984). Moguel and Toledo (1999) proposed a typology of five types of coffee production systems applicable in Mexico which is now widely cited to illustrate coffee production system diversity worldwide. Three of the types are mixed species plantings—"rustic" with coffee planted into thinned forests, "traditional polyculture" with coffee and a diversity of other useful plants planted into thinned forest and "commercial polyculture" with coffee and a diversity of useful trees creating a multi-strata vegetation complex. The remaining two types have simplified tree species and structure with only one or two tree species or without trees to maximize coffee production *via* monoculture. The gradient has been generated by coffee growers seeking greater cash income in response to technological and development forces aimed to improve coffee yields through increasing monoculture.

The drawbacks of smallholder livelihood strategies dependent on coffee alone are highlighted for Central America and Mexico (Fernandez et al., 2013; Bacon et al., 2014; Anderzén et al., 2020) as a larger issue for food sovereignty and sustainable development in rural communities. Increasing seasonal food insecurity measured as "lean months" is associated with greater percentage of household land in coffee, declining on-farm production of annual food crops and low-income diversification (Morris et al., 2013). Pinoargote et al. (2017) in their comparison of three coffee production systems of increasing diversity found that coffee was the main income in all three systems. Total benefits were similar in financial equivalents across systems, but more diversified and with greater carbon accumulation for the systems with higher plant diversity. They sampled only fields with well-managed, productive coffee and emphasize in their conclusion that good coffee management, pruning and adequate inputs are key to achieving these system synergies, although they did not address household food security. In a coffee growing region of Ethiopia, Duguma et al. (2019) documented four production systems. The annual food crop systems were much less diverse in species and had fewer ecosystem services overall, although the provisioning function of food production increased. Three systems generated coffee income—two types of coffee agroforestry and home gardens which had both coffee and high species diversity, including some for food consumption (**Table 1**). Fernandez and Méndez (2019) also contrasted home gardens (primarily without coffee), annual food plots and coffee fields on 79 farms in Chiapas, Mexico. Household food security was linked to on-farm diversity. Although major dietary components came primarily from annual food plots and home gardens, they also identified leafy green vegetables which were collected from coffee fields, highlighting the potential to increase food security benefits from the coffee plots themselves.

In our own field work on coffee agroforestry in Central and South America, we documented tree species diversity and stem density, quantified tree and soil carbon and income mostly focusing on bananas in the system (Staver et al., 2010; Cerdán et al., 2012; Siles et al., 2012). However, we have not addressed the multiple benefits potential of traditional diversified agroforestry as a smallholder strategy with both profits from global coffee markets and food security, income diversification, on-farm inputs to production and other livelihood needs. In this paper, we build on our own field experience and a survey both of coffee sector documents and academic research to propose an

TABLE 1 | Selected parameters from studies of smallholder coffee multi-strata diversity in four continents (nr, not reported by authors).

Continent	Country/region	# trees /ha	# coffee plants/ha	# banana stems/ha	Tree Shannon diversity	Products from associated vegetation	References
Asia Indonesia	Lampung, Sumatra	200–350	1,200–1,550	None	Nr	Timber, firewood, legume, fruits, spices	Philpott et al., 2008
Asia Indonesia	Western Lampung	97–350	nr	nr	0.8–1.4	Timber, legume, firewood, fruits, spices	Evizal et al., 2016
Asia India	Western Ghats	273–377	nr	nr	2.68–2.71	Timber, legumes, firewood, native tree diversity	Ambinakudige and Sathish, 2009
Asia India	Kerala	nr	nr	None	Nr	Timber, firewood, native tree diversity, black pepper, ground spices	Kumar et al., 2019
East Africa Ethiopia	Southeastern: Harerge	nr	nr	None	Nr	Legume, timber, fruit, stimulant, grains, pulses	Teketay and Tegineh, 1991
East Africa Ethiopia	Southwestern: Yayo Coffee Forest Biosphere	nr	nr	Banana, Ensete	3.14	Timber, legume, firewood, fruits, tubers, fodder, vegetables	Duguma et al., 2019
		nr	nr	None	1.21	Timber, wild fruits, legume	
		nr	nr	None	2.09	Timber, wild fruits, legume	
East Africa Uganda	Mt Elgon	63–146	2,200	10–240	Nr	Timber, firewood, fodder, fruits	Rahn et al., 2018
East Africa Tanzania	Mt Kilimanjaro	100	nr	1,000	Nr	Timber, fruits, legume	Wagner et al., 2019
West Africa Togo	Atakora mountains	198–273	2,670	None	3.58–4.06	Timber, legume, fruits, cloth, food	Koda et al., 2019
West Africa Cameroon	Western Region Kekem, Haute-Nkam	133	nr	650–1,000	0.5–2.16	Oil, legume, timber, fruits, medicinal	Temgoua et al., 2020
C America	El Salvador, Honduras, Nicaragua	198–488	Nr	10–240	1.57–3.08	Legume, timber, fruits	Somarriba et al., 2004
C America Nicaragua	El Cua, Tuma-La Dalia, Rancho Grande	114	5,356	516	1.23	Legume, firewood,	Pinoargote et al., 2017
		197	5,691	208	1.69	Legume, firewood, timber	
		437	5,946	179	2.36	Legume, firewood, timber, fruits	
N America Mexico	Chiapas, Mexico	371	1,500	nr	Nr	Native trees, timber, legume, firewood, fruits, materials other uses	Soto-Pinto et al., 2001
Caribbean Dominican Republic	Mixed with varying proportions of fruits, service, timber, banana tubers	539	3,732	450	Nr	Avocado, citrus, tubers, firewood, banana	Tapia et al., 2021
		412	3,418	301		Citrus, timber, avocado, firewood	
		853	3,366	738		Banana, citrus, timber, firewood, tubers	
		534	3,731	417		Timber, other fruits, cocoa	

initial hypothesis and a multiple benefits scoring framework. We test the framework using data collected previously of species diversity, stem density and biomass in coffee agroforestry in Nicaragua. Our conceptual timeline based on three scenarios to increase benefits illustrates the tree species and stem turnover in response to numerous market and livelihood challenges.

We hypothesize that while current smallholder agroforestry substitutes for certain coffee production inputs and provides opportunistic livelihood benefits generating stable participation in an unstable global coffee market, a transformation is opportune to more active management of species and stem turnover in system renovation cycles targeted to generating greater and more diverse benefits to farm household income, livelihood needs and ecosystem services to coffee production.

The phases of our analysis were as follows with greater methods details in the respective sections:

1) survey of research and technical documents in response to three questions: Does traditional tree diversity translate to critical livelihood benefits? Are practical research results increasingly available to build strategies for more benefits-oriented diversity in coffee agroforestry? Are the drivers of global markets compatible with more benefits-rich smallholder coffee agroforestry?
2) design of multiple benefits scoring framework drawing on insights from the document survey and test using our previously collected data sets of tree species density in 140 coffee fields in Nicaragua to visualize how tree

use characteristics and density converts into market diversification, seasonal food needs, other household uses, coffee productivity maintenance and habitat;

3) feasibility testing of benefits intensification scenarios by grower typology to address recurrent gaps like food security and income diversification scored with the same preliminary system. The program SExI- FS which models light capture and penetration based on individual tree canopy dimensions and characteristics was used to target at least 50% light for high coffee productivity with different reconfigurations of the agroforestry components.

In the discussion we address gaps in current data on traditional agroforestry systems and highlight the need for simple methods to collect data on-farm of the current benefits of trees by species, tree specifications and density. Equally the benefits potential of alternatives needs to be data-based. Such data have relevance for academic hypothesis-testing and data-driven grower strategies for agroforestry transformation.

DOCUMENT SURVEY: THE FUTURE FOR DIVERSE, BENEFITS-RICH SMALLHOLDER COFFEE AGROFORESTRY

Coffee agroforestry used by small growers has evolved and developed over the past 100 or more years in a context quite different from the forces at work currently. Our three-phrase document survey summarized in this section addresses first the benefits offered by smallholder coffee agroforestry reported in academic literature. Next, we profiled the contribution of coffee research to the challenges of future agroforestry systems, both component and system research. Finally, we surveyed academic work, sector strategy papers and advocacy proposals to characterize market, demand and regulatory pressures which might influence the transformation of coffee agroforestry.

In the following sections we provide substantiating detail contributing to our hypothesis. In summary, traditional low yield, multi-strata smallholder fields will need to undergo modification to become a viable livelihood for the next generation of smallholder growers and workers. An increasing demand for fine and bulk coffee ensures an available market. Agroforestry offers practical advantages for income and food diversification, and for input efficiency and reduced externalities.

However, on-going species and variety substitution to address climate change and market demand will be needed with altered species interactions to be addressed through ecological intensification as a pathway for more cost-effective coffee production. Driven by certification standards and national regulations, future smallholders of agroforestry coffee will need to address benefits such as habitat for native flora and fauna and clean water. Socio-economic, production and ecological research inputs will be needed to support updated multi-objective smallholder coffee agroforestry. Practical data collection and analysis tools can leverage the strengths of traditional agroforestry for data-driven transformative management for greater ecological intensification and economic efficiency.

An Overview of Local Benefits From Smallholder Coffee Production Systems

Studies worldwide inventorying the plant diversity in established coffee fields document farmer strategies to achieve cash income from coffee, while also addressing local benefits (**Table 1**). The cases selected by a search for studies with tree density and use illustrative of major continental coffee production regions present data from different plot sizes and different minimum tree diameters and calculate indices based on data sets with different samples—sometimes plots or multiple plots in a single community. Few studies quantified overhead shade and coffee productivity. We report the data as cited in the papers which provide an overview on farmer innovation in multi-strata coffee production in response to local biophysical, market and home consumption contexts. In all plots coffee is the key income source and a major plant component. Trees in the plots referenced from Asia, East and West Africa, Mexico, and Central America and the Caribbean, ranged in density from 50 to as many as 350–800 stems/ha. Wood products—timber and firewood—appear with greatest frequency followed by nitrogen-providing legumes and fruits. Shade, soil protection and habitat are not mentioned and are assumed, based on the presence of trees, to be services of all the plots listed. The lower tree diversity in certain zones in Asia provides these basic services, but little in terms of food and alternative income, while in higher diversity systems such as West and East Africa and some regions of Latin America the provision of food, income and other household materials is more common. Native tree and shrub species are an important component in India, Togo, Ethiopia, and Mexico. Zones in East Africa, Central America, and the Caribbean incorporate abundant banana mats as a major food and income crop in the agroforestry system. The presence of annual food crops is documented for cereal grains, pulses, tubers and vegetables in southeastern Ethiopia and tubers for coffee agroforestry plots in Dominican Republic.

On balance, the rich documentation of smallholder multi-strata coffee fields in **Table 1** suggests that the presence of trees generates certain services due to the nature of trees as a growth form—shade, soil protection, and wood products for fuel and local construction. Other benefits depend more on the composition of associated tree species—nitrogen enrichment, fruits, and spices. Staple foods are a relatively uncommon benefit in the studies cited. The range of stem density and species diversity reflected in **Table 1** is the source of abundant non-food local benefits compared to sun-grown coffee or with shade from a single tree species. The conversion of coffee agroforestry to annual food or income crops represent lower plant species diversity and a loss of associated services, although increased food (Duguma et al., 2019; Fernandez and Méndez, 2019). On the other hand, the expansion of coffee agroforestry at the expense of land for food production may result in losses in local food autonomy (studies already cited from Central America and Mexico), although accompanying gains in services like carbon, soil cover, water conservation, and habitat. This contrast of coffee agroforestry systems globally generates a question—how can traditional coffee plot tree diversity be oriented toward greater local benefits like food security and income diversification?

Trends in Research on Coffee Agroforestry Improvement

In this section we highlight advances in three major research lines—varietal development and pest and disease management, coffee agroforestry system description and improvement, including participatory research and economic efficiency analyses. We also identify gaps and opportunities for further research based on the other two sections of this document survey and our own experience.

Globally coffee research directs major efforts to diseases and pests, in particular coffee rust (Avelino et al., 2018) and coffee berry borer (Jaramillo et al., 2006) which generate both increased costs of production and often large losses for growers of all sizes. Complex food web interactions affecting losses to coffee pests and diseases have also been documented (Perfecto et al., 2014) in diverse agroforestry, although integrated management strategies incorporating these interactions are still under development. New coffee cultivars are in the pipeline to address disease losses, although primarily for lower shade and higher fertilizer inputs (Vossen et al., 2015). Varieties for agroforestry systems also incorporating climate change and coffee quality are the target of multi-partner breeding program (Bertrand et al., 2019). Such component research results on varieties for climate change and coffee cup quality under agroforestry and pest and disease management are essential to improving the viability of future smallholder coffee production enterprises.

The multiple objectives of smallholders in growing coffee with multi-strata trees are also a topic of research. Studies of existing diversity have already been summarized in the previous section. Staver et al. (2001) proposed an integrated system perspective to design pest suppressive multi-strata. Allinne et al. (2016) expanded on pest damage interaction with system characteristics as quantified in other ecosystem services. However, their study addressed highly contrasting production strategies with limited data on differences among agroforestry strategies. Tree canopy models have been developed to project light capture and penetration in mixed or single species tree plantings based on canopy dimensions, canopy openness, tree height and location with GPS coordinates (Harja and Vincént, 2008; Quesada et al., 2010). However, they have not been applied in highly diverse tree stands. More advanced models like Maespa are oriented to modeling ecophysiological processes including coffee in relatively simple agroforestry systems (Vezy et al., 2018). Research groups such as Rahn et al. (2014) have analyzed trade-offs and synergies of contrasting coffee agroforestry systems on-farm primarily from the perspective of carbon capture, climate change adaptation and mitigation and livelihoods. They use tree inventories of current systems to suggest best practices. The effects of climate change on tree species in coffee agroforestry were highlighted by de Sousa et al. (2019). They conclude that many current tree species in coffee agroforestry will not be adapted to higher temperatures and classify the ecological niches of 100 tree species in Mesoamerica to guide species substitution as climate continues to change.

Participatory research approaches have also addressed adaptation to climate change and improved food security with relatively little input from science and research (Shapiro-Garza et al., 2020), while others such as Cerdán et al. (2012) and Gram et al. (2018) have re-interpreted local knowledge in an ecosystem services framework. These approaches are deployed to facilitate farmers into action in a project framework with low potential to address the challenges of agroforestry redesign and management toward more targeted local benefits.

Economists have looked at the efficiency of coffee farms in different contexts (Binam et al., 2003; Perdomo and Mendieta, 2007; Ngango and Kim, 2019). The studies have primarily addressed technical efficiency of coffee production with results suggesting that coffee farms in Rwanda (82%) convert inputs into outputs more efficiently than farms in Cote d'Ivoire (47%) and Colombia (42%). The approach uses survey data from farms to generate production functions suggesting that the technology across farms in Rwanda is much more uniform than in Colombia. The study of coffee farms in Colombia expanded the analysis to contrast three farm sizes which addresses scale efficiency and allocative efficiency—the mix of land, labor and capital. The authors found that large farms deployed more optimum input levels, but that medium and small farms were lower cost producers. They also concluded that the coffee sector in general offered much scope for improvements in economic efficiency. The study in Cote d'Ivoire also identified significant scale efficiencies suggesting that farms could become more efficient by modifying the size of their operation. The authors identified both positive and negative socioeconomic factors linked to technical efficiency differences. For example, large family size may result in overuse of labor beyond efficient levels. None of these studies provide technical parameters on labor or input productivity, the role of tools, equipment or means of transport on-farm, tree species, age or pruning strategies, agroforestry composition, age of planting or renovation strategies, availability of off-farm services or resource endowment like clean water sources or road access. Ofori-Bah and Asafu-Adjaye (2011) focused on cocoa agroforestry to study efficiencies of scope, including intercropping and shade trees in their analysis, The efficiency of deployment of land, labor and capital in environmental sustainability has been measured (Grzelak et al., 2019) and environmental measures were incorporated into an analysis of cocoa in Indonesia by Tothmihaly et al. (2019), but no studies are available for coffee production. The long-term data base of farms in Europe from Grzelak et al. (2019) suggests that larger and more capital-intensive farms generate environmental value more efficiently. Practical calculations on the environmental costs of coffee production (https://trueprice.org/monetisation-factors-for-true-pricing/) conclude that the current price does not cover the environmental costs, but studies are yet to be done on farm to farm variability and the role of coffee agroforestry, worker skill sets and income levels, coffee processing technology, and other factors in coffee production eco-efficiency.

A large applied research challenge emerges from this brief analysis of technical, scale, allocative, scope, and eco-efficiencies which is largely economic in nature built from existing farm data. Production scientists, agroecologists and economists using farm and field data along with technical and ecological parameters could formulate and test alternative coffee agroforestry scenarios

based on different factor endowments, farm sizes and production technologies and ecological processes. A potential product of such efforts would be practical data collection tools for smallholders to manage data-driven diversified agroforestry coffee from an enterprise perspective with accounting for externalities. Contreras-Medina et al. (2020) take a value chain stakeholder perspective to "roadmap" key technologies and capacities to a more sustainable future for the coffee sector in Chiapas, Mexico. They combine surveys of coffee farms and foresight analysis on emerging technologies and market demands to develop their conclusions which do not include coffee agroforestry. However, they highlight the need for a better understanding of small farm needs, the nature of emerging technologies and potential for the use of digital tools. In a major study on global coffee sustainability, Sachs et al. (2019) make no specific mention of agroforestry in their recommendations for national coffee sustainability plans. Similarly to Contreras-Medina et al. (2020) they take a commodity focus rather than a farm enterprise approach.

In summary, current research efforts are directed primarily to improved coffee varieties, including for agroforestry systems and climate change, and pest and disease management. Studies on coffee agroforestry as illustrated in **Table 1** document species diversity of current farmer strategies, while other studies not cited here quantify carbon storage which we considered a global and not a local benefit for climate change mitigation. While economic analyses are limited, they illustrate an important dimension to science inputs. We propose that research should be expanded to address the design and management of future smallholder coffee agroforestry fields and enterprises from the perspective of farm and value chain profitability, including worker wage livelihoods, sustainability, and resilience incorporating production technology, ecological intensification and technical, allocative, scale and eco-efficiencies.

Drivers of Future Coffee Production Systems and the Potential for Local Benefits

A summary of driving forces which will influence the viability of the next generation of smallholder coffee farms is a key input into strategizing agroforestry alternatives which update system benefits in evolving global and local contexts. While a more thorough foresight analysis of future smallholder coffee production systems would be opportune, here we summarize four areas of relevance from the market perspective—demand and pricing, enterprise competitiveness, certification and regulation requirements and climate change/pests and diseases which affect global supplies.

Demand for coffee is growing, both specialty coffee and bulk coffee for coffee-based products (Panhuysen and Pierrot, 2014, 2018, 2020) which may have quite different structures of costs of production and marketing. Higher prices of export gourmet coffee may be more appealing, but the production of bulk coffee with lower production costs may offer lower risk income and greater flexibility for agroforestry systems. Production for national markets may offer production options to regions with limiting abiotic conditions for high quality coffee. Around a third of global coffee production is consumed in producing countries and future growth in coffee consumption will occur primarily in developing countries (Sachs et al., 2019). Coffee certified under different labels may also offer price incentives suitable for certain groups of growers with potential demand limitations on specific labels. For example, De Janvry et al. (2011) calculated that the price bonus in FairTrade coffee will result in overproduction and a loss of benefit to growers precisely because demand is limited, and coffee cooperatives are not able to place all their production with FairTrade buyers. While overall the future of coffee may appear promising, several studies (Earth Security Group, 2017; Sachs et al., 2019; Panhuysen and Pierrot, 2020) warn that the current coffee business model is not sustainable due to high environmental costs and low commodity prices which are unprofitable for smallholder producers who produce up from 60% of global production.

Small farms as enterprises must survive in an uncertain and evolving global coffee market. Numerous studies, summarized by Muñoz-Rodríguez et al. (2019), suggest that currently the costs of production of coffee are greater than prices paid and do not provide a living income. The True Price method (https://trueprice.org/monetisation-factors-for-true-pricing/) also includes externalities driving the actual cost of conventional coffee even higher. Barham and Weber (2012) found that yields rather than certification were more important to increasing net household returns from coffee production. They conclude that certification schemes should incentivize yield improvement addressing both grower wellbeing and compliance with certification norms. They highlighted the role of fertilization in yield improvement. Hernandez (2020) proposes a smallholder model primarily with family labor for Mexico with inputs to achieve yields of 800 kg/ha/year on 3 ha of coffee agroforestry. Pinoargote et al. (2017) also proposed that adequately fertilized and pruned coffee is a cornerstone to diversified multi-strata approaches. Their three grower groups reported yields from 500 to 850 kg/ha/year, above national averages in many countries. Coffee harvesting is a major cost component with a lower cost per volume harvested in higher yielding fields, illustrative of the importance of higher yields. Seasonal workers seek out farms with higher yields to contract their services paid by piece work and growers often complain of labor shortages. Fields with sparse and dispersed berries provide less income per day to workers than high yielding bushes. The labor challenges are illustrated vividly by Jimenez-Soto (2020) who documented the farm worker perspective in current coffee production and Cofre-Bravo et al. (2019) who identified farm workforce engagement in the innovation process as a key variable in the most dynamic enterprises. At the same time, the diversified livelihoods approach of smallholder coffee farms has been documented and has great relevance for an uncertain future (Anderzén et al., 2020). In their evaluation of the sustainability of the coffee sector, Earth Security Group (2017) identifies the need for "regenerative production systems which are commercially viable," although they do not mention food security or income diversification. Improved yields/ha, for example, a doubling

of national yield averages, may benefit efficient growers, but would lead to general overall overproduction putting downward pressure on coffee prices (Waarts et al., 2019). Well-designed multi-strata agroforestry appears to offer a way forward based on well-yielding coffee with lower costs of production based on nutrient recycling and nitrogen enrichment, pest and disease suppression based on biotic and abiotic factors and labor efficiencies in harvesting and other practices. At the same time, drawing on lessons from traditional diverse multi-strata systems, well-selected and managed agroforestry system components could provide targeted complementary income diversification and production of staple foods ensuring enterprise resilience in the face of price fluctuations and climate and pest/disease variability.

Beyond enterprise issues, future smallholder coffee systems also face ever increasing requirements/opportunities from value chain certification frameworks through price incentives and national and local policies and regulations which orient food, worker and environmental safety through legal compliance mechanisms. Lambin et al. (2014) refer to the demand-driven voluntary certifications which enable access to certain markets and the command and control of national regulations. For coffee, Millard (2017) identifies three demand-driven initiatives—certification frameworks, company-driven standards and industry platforms. The certification frameworks in coffee are organic, FairTrade, Rainforest, and Smithsonian Bird-Friendly which have often entered into alliances with companies like Starbucks and Nespresso which have their own certification procedures. The 4-C certification platform originated from agreements among large food corporations to cover more basic environmental, social and economic issues. Specifications on multi-strata composition and structure are found primarily for bird-friendly coffee, while the other certifications have more general requirements around biodiversity, deforestation, soil and water management and social and labor criteria primarily to generate consumer confidence. A meta-analysis of studies on the contribution of voluntary certification on sustainability suggested that some evidence exists for positive impact primarily measured on environmental factors and price received, although not grower income (DeFries et al., 2017). Vellema et al. (2015) concluded that the extra costs to meet certification standards reduced income from other non-coffee activities resulting in no increase in overall household income from certified coffee production. Bianco (2020) examined corporate social responsibility strategies to address climate change in coffee and concluded that certification should be complemented by more direct action in development of alternative varieties for substitution and on-farm investment. The precise nature of certifications and standards and their approaches to both consumer and producer expectations is uncertain, but will certainly become more rigorous and directive in the next years. Future generations of smallholder multi-strata coffee producers and their organizations will need to be versatile, creative and data-driven managers of certification requirements to leverage them to guide small farm viability compatible with coffee agroforestry.

The command-and-control regulations by national governments for the environment, labor safety, use of pesticides, and food safety will also orient smallholder coffee production systems toward greater compliance with worker safety in such activities as tree pruning and pesticide use and more careful management of coffee wastes and increased infrastructure in coffee processing to reduce pollution. For multi-strata coffee, regulations on tree cutting will influence farmer willingness to manage their tree planting and cutting for timber and other benefits. Detlefsen and Scheelje (2012) concluded based on a comparison of regulations in the countries in Central America that legal procedures for tree cutting were very variable and only in few countries were simple and well-implemented to make on-farm planting for extraction attractive. Regulations on transparent and effective tree extraction and other core activities of coffee agroforestry may still be in the future in most coffee-growing regions. However, small coffee agroforestry enterprises and their associations will need to address the paperwork and investment from increasing labor, environmental, and business regulations.

Among biotic and abiotic factors threatening coffee returns and smallholder livelihoods are the resurgence of coffee rust and the breakdown of resistance in currently resistant varieties (McCook and Vandermeer, 2015), the possible spread of new diseases not yet present in certain regions like coffee berry disease and coffee wilt (Waller et al., 2007) and an increasing erratic climate linked to global warming (Morris et al., 2016; Pham et al., 2019). Abiotic and biotic threats may also threaten the associated plant diversity in the multi-strata system. For example, service trees currently used in multi-strata coffee may be less adapted to higher temperatures (de Sousa et al., 2019). These threats can be addressed with multi-strata production which offers micro-climate modification and increased beneficial flora and fauna. However, the traditional system based on a relatively static composition of certain cultivars and species will need to undergo dynamic evolution with change in coffee varieties and associated timber, service and fruit trees under the active management of growers backed by other grower experiences, technical assistance and scientific knowledge.

Salient characteristics of future smallholder coffee agroforestry emerge from this survey of drivers. "Regenerative production systems which are commercially viable" (Earth Security Group, 2017) will need to include moderately high yielding coffee and multi-strata agroforestry with components which are selected and managed to provide multiple and targeted benefits—reduced coffee production costs, diversified income and sustained support to household livelihood needs. In terms of grower management tools, data demands both for certification and national regulations should be leveraged to facilitate more dynamic grower adaptive management in terms of efficiency, resilience and profitability. This suggests that growers will move from the continuity and stability of traditional diverse agroforestry to more dynamic benefits-oriented use of diversity with species and stem turnover to optimize contributions to coffee productivity, income and household needs and to respond to shifting climatic and market conditions.

ADDRESSING BENEFITS GAPS IN COFFEE AGROFORESTRY: AN APPROACH AND PRELIMINARY TEST

Previous sections suggest that tree species diversity common in smallholder coffee agroforestry must be analyzed in terms of specific livelihood benefits. Documenting diversity alone is not sufficient. This step is fundamental toward proposing targeted modifications which address livelihood benefits gaps. In this section, we present our test using previously collected data to focus on the recurrent gaps in local benefits which are threatened by the shift to coffee monoculture or simplified agroforestry. The illustrative exercise tested our hypothesis and provided us insights and priorities for a research and development agenda addressing livelihoods benefits intensification in coffee agroforestry. In the following section we first describing a multiple benefits scoring framework. After providing details on our methods, we characterize our data sets from 140 coffee agroforestry fields and apply the multiple benefits scale to four typologies grouping smallholder coffee plots in the data sets. Finally, we analyze three scenarios for benefit enrichment compatible with improved coffee yields applied to examples from each typology.

A Method to Characterize Benefits Gaps in Smallholder Coffee Agroforestry

Multi-functionality is a characteristic of all ecosystems, although the measurement and comparison across functions may present challenges (Garland et al., 2021). Our concern here is to test whether ecosystem services generated by ecosystems functions result in livelihood benefits to coffee growing households and their communities. Our studies on the optimization of banana in coffee multi-strata agroforestry have provided insights into both multiple benefits and their distribution during the year (Staver et al., 2010). These benefits from banana include compatibility with mixed species systems, ease of establishment and management for shade and high biomass production for soil protection and improvement. Farm households are very clear in the importance of the monthly income resulting from the sale of bananas as a petty cash fund. Bananas are a food staple in a few coffee-growing regions and in other regions provide food for lean months and backyard animals. Leaves and stems are used in food preparation and as temporary shelter and packaging material. Banana flowers available in most months of the year depending on rainfall distribution attract a wide diversity of insects and ripening bunches of bananas left in the field attract mammals and birds. The banana example suggests certain dimensions important to screen alternatives to address gaps in local benefits. The reviews in the previous sections point to similar issues generated by the shift to less diverse coffee agroforestry and open sun monoculture described by Moguel and Toledo (1999). First, the "lean months" refer to both food and income shortfalls in specific months of the year. Second, dependence only on coffee income with volatile prices results in livelihoods vulnerability. Third, the reduction of ecological functions from agroforestry in support of higher yielding coffee production are not available from coffee monocultures or agroforestry with few species and need to be replaced with external inputs. Finally, biodiversity loss relating to the first three issues generates a loss of habitat functions.

Studies of species present in coffee agroforestry have quantified ecosystem services. Duguma et al. (2019) convened farmers in the Yayo Biosphere to rate four land use systems for 15 ecosystem services defined in the Millennium Ecosystem Assessment. This approach did not link tree species to the services which reduces its utility for multi-strata redesign. They looked at trends over decades, an important perspective, but did not capture within-year provision of benefits. Gram et al. (2018) convened farmer focus groups to document their classification of specific tree species in terms of regulating and provisioning services. The resulting information provides a rich basis to identify alternatives for increased services. They did not include income generation among the services and did not capture the within-year distribution of services which our experience with banana suggests is an important criteria for farm households. Cerdán et al. (2012) also documented farmer knowledge of tree species services focusing on services to coffee and soil productivity and habitat for biodiversity, missing food provision, income diversification and the within-year dimension.

We propose the framework described below to focus on recurrent gaps in livelihood benefits. We assume that soil and water conservation, leaf litter and micro-climate modification are general services from all species or are more related to number of individual trees, their size and layout. This assumption could be modified in later versions of the approach or for more location-specific analysis. To target the selection process on gaps, we rated each species according to five categories of benefits—coffee productivity, income, food, other household uses and habitat with sub-categories for each of the five types. We also rate species according to the strata they generally occupy into three categories—upper (trees), midstory (trees and bananas), understory (coffee, bushy, and herbaceous species) which contributes a useful filter to screening alternatives. We emphasize that our sub-categories are exploratory to illustrate the approach. The scales below are proposed to classify each species present in the coffee agroforestry plot:

- **Coffee productivity:** 0 – none; 1 - nitrogen fixation; 2 - temporary shade; 3 – phosphorus accumulation. The more general benefits of trees are not included in this rating. Leaf phenology might also be the basis to characterize differences in tree species, although in actively-managed mixed species multi-strata systems, some degree of shade will always be overhead. Another sub-category might be presence of nectaries (Rezende et al., 2014). This potential benefit could also be addressed through an additional category of service linked specifically to micro-environment effects and biocontrol of pests and diseases of coffee and other plant species in the multi-strata system.
- **Income:** 0 – none; 1 - monthly; 2 – seasonal for export; 3 – seasonal for national markets; 4 – seasonal for local sale and exchange; 5 – multi-year. Information on the season of production and the target market captured in these

sub-categories is an important input to income diversification. Rice (2011) provides useful insights about the characterization of fruit production in the household economy. Banana harvest, for example, although fluctuating throughout the year in response to abiotic conditions, still produces bunches in most months, even with rustic management.

- **Food:** 0 – none; 1 - monthly; 2 – seasonal perishable; 3 – seasonal storable; 4 – emergency seasonal. The seasonality of food availability is highlighted in the studies on the lean months (Fernandez et al., 2013; Morris et al., 2013) suggesting that this benefit should have sub-categories by season. We also envisioned differences between perishable fruits and stored products like seeds and emergency foods which are sourced primarily in times of stress. We have seen that poorly filled out banana bunches can become food in years when lean months are severe.
- **Other household uses:** 0 – none; 1 - firewood; 2 – construction material; 3 – resins and medicinals; 4 - fibers and coverings. We debated whether this should be by season or by product and concluded that many of these are available year round with the exception of medicinals and resins which may be non-perishable. This last use is listed commonly in **Table 1** as well and merits more thorough data collection.
- **Habitat**: 0 – none; 1 – birds; 2 – bats; 3 – bees; 4 – small mammals. Peters et al. (2016) argue that tree species selection should be guided by type and season of feed resources provided to fauna. Narango et al. (2019) and Chain-Guadarrama et al. (2019) provide more specific information on insects as feed resource for birds and bees in pollination. We included this benefit based on types of fauna, but realize that both our own experience and the literature are limited to provide thorough information either by species or seasonality. Few tree species do not provide some habitat service, even just roosting or flowers and seeds. A scoring scale might recognize species with special habitat services either special habitat contributions for key fauna highly valued or broad general resources to multiple types of fauna and in multiple seasons.

Methods Used to Test the Benefits Gap Framework

To explore the potential of the scale proposed above to quantify local benefits, we re-analyzed three data sets from our previous work with smallholder coffee farmers in Nicaragua. The data set collected in 2009 from Monterrey (Jinotega department) and Yasica Sur (Matagalpa department) inventoried all stems > 5 cm in diameter in two subplots of 25×25 m in 30 coffee fields (Siles et al., 2012). The data set collected in 2011 from El Cua (Jinotega department) and Jalapa (Nueva Segovia department) inventoried all stems >3 cm in diameter in one 20×50 m plot in 40 coffee fields (Cerdán unpublished). The data set collected in 2016 from the Jinotega municipality (Jinotega department) inventoried all stems >2.5 cm in diameter in one 20×50 m plot in 70 coffee fields (Kichline, 2017). In each plot, all trees and saplings were identified and measured (DBH and height) for individuals above the minimum circumference at breast height (DBH taken at a height of 1.3 m). When buttresses and other irregularities were present at 1.3 m, the stem was measured 50 cm above the protuberances. For multi-stemmed individuals, DBH of all stems was measured as for the single individual. Tree species were identified by the authors in the field or with the help of dendrological keys (Gentry, 1993; Holdridge and Poveda, 1997; Zamora et al., 2000, 2003).

Our preliminary test using the scale in the previous section was carried out in three steps: (1) recombination of three data sets into typologies of agroforestry strategies based on stem densities and basal area by species; (2) local benefit quantification based on species stem density and use characteristics; and (3) analysis of three scenarios applied in one field from each of four typologies to address gaps through simulation with a spatial model and recalculation of summary statistics.

In the first step, the density of trees and saplings was determined by counting the number of individuals in the sampling plot, the stem basal area of each individual was calculated using the DBH and the plot basal area was the sum of all individual stems expressed per plot. Species richness per plot and Shannon diversity index were calculated using the BiodiversityR package in R, Version 3.2 (Kindt and Coe, 2005). Species accumulation curves (100 randomizations without replacement) were calculated using the Vegan package (Oksanen et al., 2007). To generate a typology from the three data sets together, we grouped species into three agroforestry components—*Musa*, *Inga* spp and other trees. Other groupings were tested, but did not generate as strongly differentiated typologies. An agglomerative hierarchical cluster analysis using the Ward-algorithm applied to a matrix of Euclidean distance coefficients between all plots using the Vegan package (Oksanen et al., 2007) was performed on the basal area, tree density and importance value index (IVI) grouped by *Musa*, *Inga*, and other trees. The IVI expresses the relative values of basal area and density by groups as follows: [relative basal area (%) + relative density (%)]/2; where the relative basal area is the basal area of each group divided by the total basal area in the plot and the relative density is the number of individuals per group divided by the total number of individuals present per plot (Galindo-Jaimes et al., 2002).

For step two, the three authors characterized the benefits provided by each one of the 122 tree species identified. We recognize that more precise and locally applicable characterization should combine both farmer and more broad-based scientific knowledge. Here we are motivated to illustrate a service gap-filling approach for further elaboration in later research. We visualized gaps in services by graphing plot by plot our five categories of services and the sub-categories in income and food. The level of each service was calculated by summing the number of individuals for each service across species present. These graphs for services were done both with and without banana. The graphs without banana reveal more clearly the profile of benefits provided by tree diversity.

In the final step we used spatial data of individual tree dimensions and distribution from four case studies of the nine plots measured (Kichline, 2017). Each of the plots was illustrative of one of the typologies (MIX, TD, ID and MD). We explored alternative multi-strata systems based on elimination

TABLE 2 | Characteristics of four typologies generated from the three data sets of composition of coffee multi-strata vegetation.

Description	Mix	MD	TD	ID
Musa density (ind 0.1 ha^{-1})	42.7 ± 2.3	43.4 ± 3.0	12.4 ± 3.8	6.4 ± 1.4
Inga density (ind 0.1 ha^{-1})	14.2 ± 1.0	4.2 ± 1.4	7.2 ± 0.6	11.5 ± 1.0
Diverse Trees (ind 0.1 ha^{-1})	15.1 ± 2.0	6.8 ± 0.7	22.4 ± 3.6	4.2 ± 1.0
Timber trees density (ind 0.1 ha^{-1})[a]	2.0 ± 0.6	0.6 ± 0.2	0.6 ± 0.2	0.2 ± 0.1
Fruit trees density (ind 0.1 ha^{-1})[b]	3.4 ± 0.9	1.7 ± 0.2	6.2 ± 1.7	1.5 ± 0.6
Rare2 trees density (ind 0.1 ha^{-1})[c]	2.8 ± 0.6	2.0 ± 0.4	4.1 ± 1.4	0.7 ± 0.3
Rare1 trees density (ind 0.1 ha^{-1})[d]	7.9 ± 1.3	2.6 ± 0.4	11.5 ± 2.5	1.8 ± 0.5
Basal area *Musa* (m^2 0.1 ha^{-1})	1.25 ± 0.08	1.22 ± 0.09	0.36 ± 0.11	0.16 ± 0.04
Basal area *Inga* (m^2 0.1 ha^{-1})	0.60 ± 0.05	0.17 ± 0.02	0.16 ± 0.03	0.48 ± 0.05
Basal area Diverse Tree (m^2 0.1 ha^{-1})	0.44 ± 0.06	0.28 ± 0.03	1.55 ± 0.32	0.27 ± 0.08
IVI *Musa* (%)	56.9 ± 1.7	73.3 ± 1.9	17.0 ± 4.0	20.0 ± 4.4
IVI *Inga* (%)	24.0 ± 1.5	9.8 ± 1.0	12.6 ± 2.8	59.7 ± 3.9
IVI Diverse Trees (%)	19.1 ± 1.8	16.9 ± 1.6	70.4 ± 4.5	20.3 ± 3.4
Species density (species 0.1 ha^{-1})	8.3 ± 0.5	5.7 ± 0.4	8.4 ± 1.6	4.8 ± 0.5
Shannon	1.25 ± 0.06	0.86 ± 0.06	1.18 ± 0.21	1.08 ± 0.11
% Shade	39 ± 19	41 ± 19	29 ± 19	41 ± 17

MIX, Inga, Musa and trees balanced; MD, Musa dominated; TD, other tree dominated; ID, Inga dominated.
[a] *Timber species found in >20 plots—Juglans olanchana, Cordia aliadora.*
[b] *Fruit trees found in >20 plots—Persea americana, Citrus spp, Mangifera indica, and Psidium guajava.*
[c] *Species found in 9–20 plots, including less common timber and native fruits.*
[d] *Species found in fewer than 9 plots, including less common timber and native fruits.*

and substitution of species with a different suite of services addressing specific gaps, while ensuring a minimum of 50% light to the understory coffee. The spatial model SExI-FS was used to complete simulations of light availability from alternative multi-strata (Harja and Vincént, 2008). The resulting stem densities by species were also scored by type of benefit as was done in step two.

Re-interpreting Coffee Agroforestry in Nicaragua in Terms of Local Benefits

Our three data sets from the northern Nicaragua coffee zone had a total of 140 tree species with 18 unidentified species (**Supplementary Table 1**). The species accumulation curves for each data set (**Supplementary Figure 1**) show minor differences which could be attributed to slight differences in sampling methods and territory covered. For example, the Monterrey/Yasica site not only covered two zones on different sides of the mountain, but also sampled two plots within each field generating a slightly higher species accumulation curve.

The typology resulting from the combined data set (**Table 2**) highlights how growers combine and recombine the suite of species common in coffee fields in the region. Three of the typologies represent fields with domination of either Musa (MD), *Inga* (ID), or other trees (TD), while the remaining typology has more equal proportions of all three groups (MIX). **Figure 1** highlights the separation of the four typologies based on the IVI scores for each plot. Each typology dominated by a single group is concentrated in a different corner of the triangle with MIX more centric representing more equal proportions of each group, although tending more toward the high Musa corner. MIX has the highest overall stem density with a higher species density and Shannon index. MD and ID had the lowest values both for species density and Shannon. The typology TD brings together higher tree density, diversity and basal area, although the group has lower timber species density than MIX. The typologies show stronger separation for Shannon index and species density than the three data sets (**Supplementary Figures 2**, **3**) which is also apparent in the species accumulation curves by typology. TD and MIX accumulate species at a faster rate and to a higher level, while MD and ID are lower on both dimensions. Average % shade of

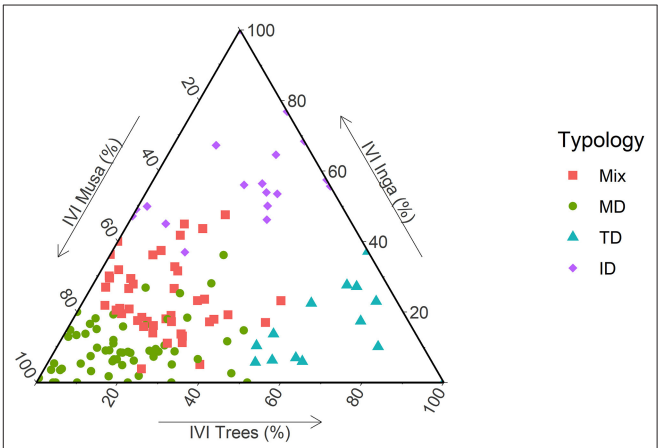

FIGURE 1 | Sampled coffee plots graphed by their importance value index of three categories of multi-strata vegetation—*Inga* (ID), banana or Musa (MD), and other trees (TD). The four typologies cluster in the triangular space based on the more dominant component with the typology MIX clustered more centrally.

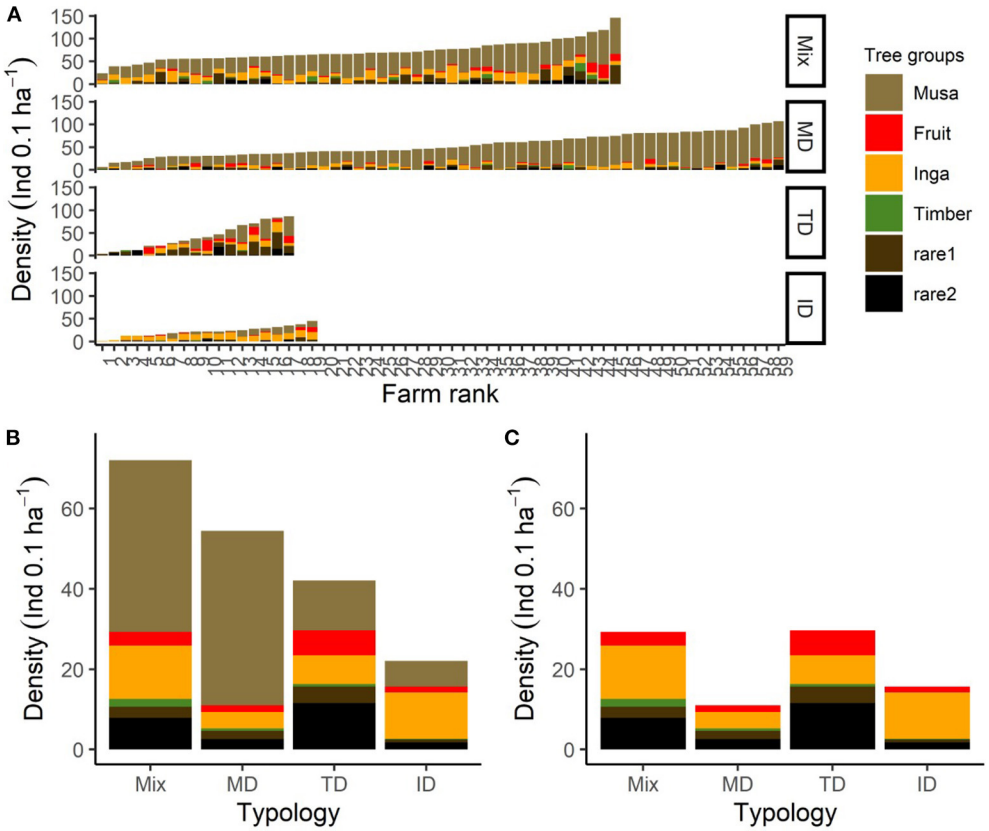

FIGURE 2 | (A) Tree composition for each of 140 plots by typology MD, ID, TD, MIX. (Fruit = fruit trees found in >20 plots—*Persea americana*, *Citrus* spp, *Mangifera indica*, *Psidium guajava*, Timber = timber species found in >20 plots—*Juglans olanchana*, *Cordia aliadora*, rare1 = species found in 9–20 plots, including less common timber and native fruits, rare2 = species found in fewer than 9 plots, including less common timber and native fruits). **(B)** Average density and composition for each typology including Musa. **(C)** Average density and composition for each typology without Musa.

40% was similar for three of the four typologies, while TD had lower shade levels at 29% with a similar standard deviation of 19% (**Table 2**). Two of the original data sets had average shade levels of 45–47% with standard deviation of 16–18%, while the data set for Jinotega had only 24% average shade. On average the typologies or data sets had sufficient light for productive coffee, but variability was also high indicating that some growers had 60% or more overhead shade in their plots, above the 50% threshold for more productive coffee (Franck and Vaast, 2009).

Plot by plot composition of individual trees by species groups for each typology highlights that within clusters there is a broad range of stem densities as seen in the overall height of each column (**Figure 2A**) from densities near zero to 100 stems or more per 1,000 m^2. Average density varies by typology (**Figure 2B**). Bananas are an important component in plots of both MIX and MD, grown primarily for markets nationally in Nicaragua or in the region in El Salvador. **Figure 2C** showing typology averages without Musa highlights more clearly contrasting proportions of the woody tree components. MIX and TD have higher tree density with greater stem density for three of the four components.

The potential local benefits available from each species found in the inventory as classified by the authors are documented in the **Supplementary Table 1**. Only seven species provide more than three of the five benefits. A common combination of four benefits, primarily leguminous shade trees, covers coffee productivity, food, firewood and habitat, while certain fruit trees generate income, food, firewood, and habitat. Only bananas provide all five benefits. The benefits provided by the fewest species were food, coffee productivity enhancement and income, while habitat and other household uses were the most common benefits. On a plot-by-plot basis, the overall range of total benefits in each typology is high from relatively few per plot to over 400 (**Figure 3**). If a plot has few individuals, quite obviously the benefits provided will be limited. Typology ID with limited banana has fewer benefits, while plots with abundant bananas have much higher benefit levels. In our simplified method to quantify benefits at plot level, a single individual may provide more than one benefit. While stem densities in **Figure 2** reach 100–150, the count for benefits reach 400 highlighting that certain stems by species offer multiple benefits. Since bananas are providers of five benefits and are also quite abundant in smallholder coffee plots in Nicaragua, they mask the benefit contribution of the remaining tree diversity. **Figure 4** contrasts average benefits by category for each typology with (4a) and without banana (4b). Without banana, total benefits

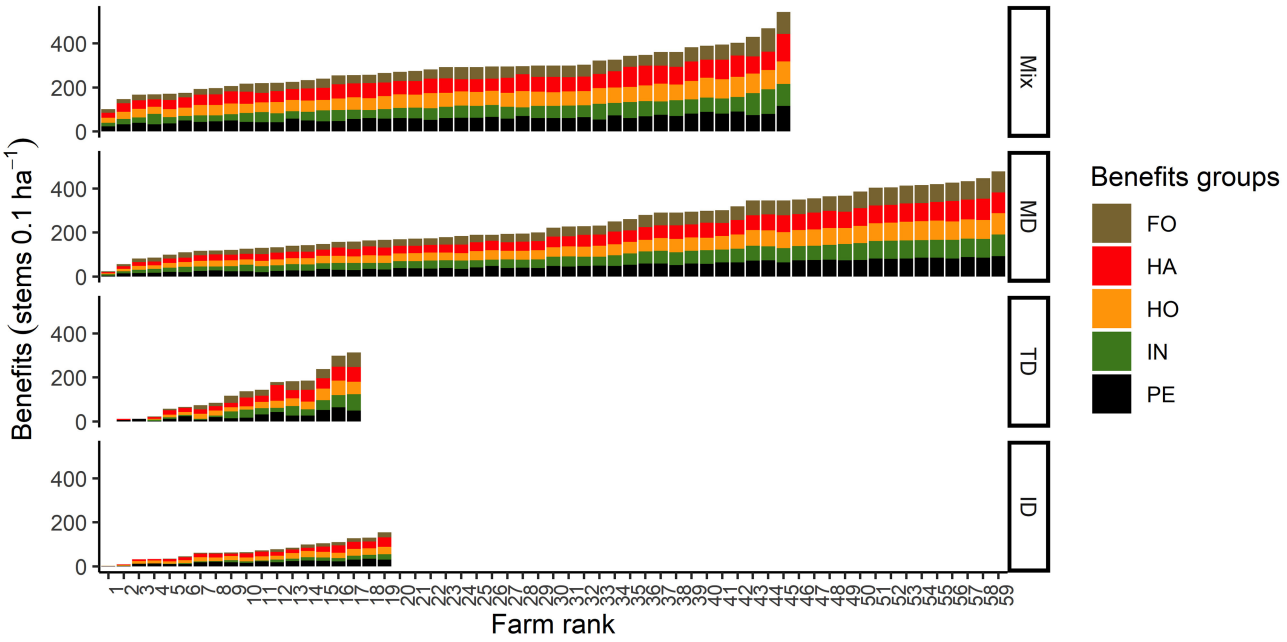

FIGURE 3 | Benefits composition from the agroforestry system in 140 plots displayed by typology. FO, Food; HA, Habitat; HO, Other household use; IN, Income; PE, Productivity enhancement coffee.

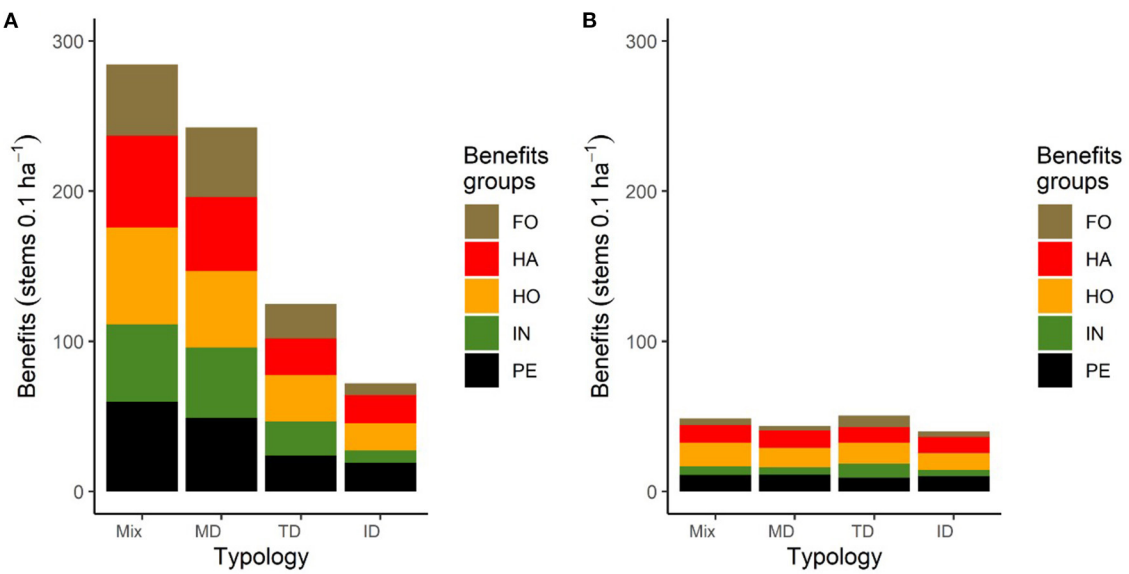

FIGURE 4 | Average benefits from the agroforestry system types, including **(A)** and excluding **(B)** banana stems. FO, Food; HA, Habitat; HO, Other household use; IN, Income; PE, Productivity enhancement coffee.

drop drastically and food and income benefits shrink as a proportion of the total. Other household uses, habitat and coffee productivity enhancement are present in all four typologies. A visual inspection of **Figure 3** highlights that the species richness of multi-strata coffee does not translate into many benefits linked to income and food. As a consequence Nicaraguan coffee agroforestry, except for banana intercropping, has only a limited contribution in addressing the lean months and income diversification highlighted earlier.

The composition of benefits by subcategory for income and food provides additional insight into the contribution of specific benefits based on seasonality and other characteristics. **Figure 5A**

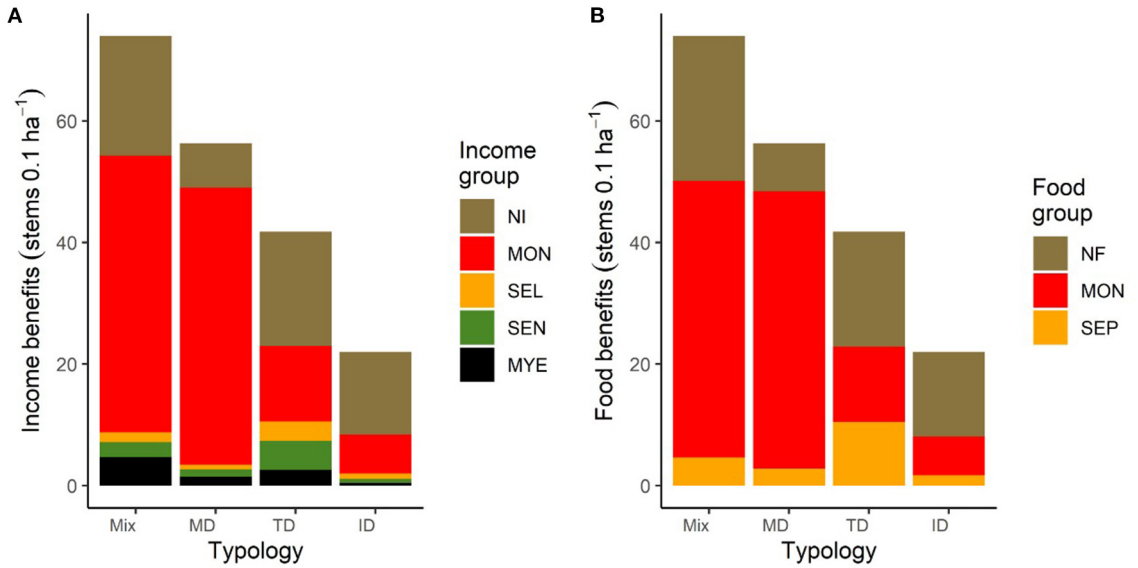

FIGURE 5 | Average income **(A)** and food types **(B)** from the agroforestry system types. Income (NI, no income; MON, monthly; SEL, seasonal local; SEN, seasonal national; MYE, Multiyear) and Food (NF, no food; MON, monthly; SEP, seasonal perishable). Categories absent from the graph are seasonable storable food and emergency or famine foods.

on income highlights that beyond the income from coffee to export markets, the monthly income from banana (MON) dominates followed by low levels of fruit (seasonal for local and national markets -SEL and SEN-) in certain typologies. Rice (2011) concluded that much of the fruit from coffee fields is wasted—not profitable for the market and too much for local and home consumption. Few benefits in **Figure 5A** result from timber, the only tree species with multi-year income potential (MYE). The timber income appears to be largely potential rather than realized income, since regulations and permissions limit tree harvest. The low density of timber trees also increases the cost of commercial extraction.

The benefits for food highlight a similar point—few species for food in only small numbers in the plots beyond banana (MON - monthly) and fruit (SEP—seasonal perishable) (**Figure 5B**). Several of our subcategories for food are not found in any plots. Seasonally produced food which can be stored such as maize or beans or even nuts and seeds is restricted to plots with a few cocoa plants. Although *J. olanchana* is somewhat common, the nuts are not consumed. No sources of emergency or famine foods were recorded, although we included it as a potential benefit from multi-strata coffee based on informal field observations during the coffee rust epidemic of 2013 when we were in the field.

Upon reflection we realized that the absence of data for seasonal stored and emergency food categories represent a methods weakness in many multi-strata tree inventories, including our own studies. Studies focus almost exclusively on the trees in intact and long standing coffee fields. In addition, most studies leave out the herbaceous component and field borders. A species like *Colocasia esculenta* is often found growing spontaneously in coffee fields with potential use only in times of dire need. The study by Fernandez and Méndez (2019) provides insight to food uses such as edible leaves from intact fields. The study of intact fields also misses the dynamic nature of coffee fields. Growers may cut down shade trees and cut back or uproot coffee during periods of low price, severe disease outbreaks or both. They may also seek to change coffee varieties or renovate shade trees. Such fields are often intercropped with annual food or cash crops providing seasonal storable food and potentially income, although data on fields in renovation or conversion do not appear in many data sets. The data set from Jinotega has 4 of 70 fields with no biomass in coffee plants suggesting recent renovation, but the other two studies missed systematic data collection on field renovation intervals and strategies both in interviews or field sampling.

In summary, the analysis of local benefits from the agroforestry component for smallholder coffee fields in Nicaragua show that beyond interplanted bananas, few other species provide food and income for the lean months or income diversification. The quantification of these benefits by subcategories provides a structure to capture distribution during the year and also differentiate between export, national and local markets. Certain authors have documented pilot programs to generate a more diversified mix of activities at the farm scale through food plots and beekeeping (Anderzén et al., 2020). In our exercise in the next section to screen alternatives for their contribution to the improvement of benefits, we direct our analysis at the scale of the coffee field, valid even if the farm is primarily planted to coffee.

TABLE 3 | Effects of scenarios on multi-strata characteristics for four cases representing MIX, MD, TD, and ID.

Descriptor	TD				MIX				ID				MD			
	Cur	Sc1	Sc2	Sc3	Cur	Sc1	Sc2	Sc3	Cur	Sc1	Sc2	Sc3	Cur	Sc1	Sc2	Sc3
Musa density	85	52	40	20	26	21	34	14	7	7	34	14	32	28	36	16
Inga density	5	2	2	0	1	1	1	1	8	6	6	0	9	6	6	0
Diverse Trees density	14	12	12	5	27	17	17	10	7	7	3	3	4	6	6	3
Timber trees density[a]	1	4	4	3	5	6	6	4	0	3	3	3	0	3	3	3
Understory density[b]	-	-	105	105 (155)	-	-	105	105 (155)	-	-	105	105 (155)	-	-	105	105 (155)
Species density	13	17	20	12	12	12	15	15	8	12	10	10	6	11	12	10
Shannon Index	0.9	1.3	1.9	1.3	1.7	1.7	1.9	1.4	0.9	1.3	1.7	1.6	1.8	2.2	1.8	1.6
Canopy openness (%)	40	44	40	78	39	49	43	76	50	54	49	77	49	51	47	78
Within-plot shade variability[c]	17	17	18	20	16	15	17	14	18	17	18	20	23	21	17	11
IN Total	91	59	152	180	54	41	159	180	7	10	142	172	32	31	144	174
FO Total	94	56	149	176	50	35	153	176	9	8	141	169	42	28	141	171

Cur, current situation; Sc1, timber with habitat; Sc2, agroforestry for zero-grazed ruminants; Sc3, rotational gap for system renewal.

[a] *Timber trees included Cedrela, Juglans, Bombacopsis, Nectrandra, Platymiscium, and Dalbergia.*

[b] *In parenthesis total including annual crops accounted at units of 10 m^2.*

[c] *Standard deviation of shade values simulated on a 2 × 2 m grid in each simulation plot.*

Testing the Potential of Diversity-Based Options to Address Recurrent Gaps in Local Services

In this section, we strategize modifications which maintain at least 50% light penetration to the coffee understory, while also generating increased benefits. Achieving improved coffee yields will require numerous additional inputs and management practices, but a base condition is adequate solar radiation for vigorous coffee plant growth. We propose this only as a preliminary step requiring numerous filters and follow-up studies involving biological, economic, and practical issues to reach field implementation. We propose that the step described here offers a concrete visualization to stimulate thinking about future smallholder coffee agroforestry with a focus on local benefits under dynamic and changing conditions. The current composition and benefits generated for income (IN) and food (FO) in each plot can be found in the columns labeled "Cur" in **Table 3**. The four cases have a broad range of shade from 50 to 60% with three cases close to the overall average of 40% with contrasting total stem densities from 220 to 1,050/ha. High banana density is found in one case. Tree density ranges from 40 to 320/ha, while *Inga* density ranges from 10 to 90. In the first step (Sc1 in **Table 3**), 50 trees/ha of five species with both timber and habitat potential or habitat potential alone with very open canopy are added or substituted depending on plot conditions. In the second step (Sc2 in **Table 3**), plots are further modified to diversify and increase monthly income and food availability. Bananas and hedges of leguminous fodder shrubs and cut and carry fodder are added to provide an adequate diet for a cow producing both milk for sale and consumption and manure to accelerate nutrient cycles. In the third step (Sc3 in **Table 3**), we simulate renovation in which coffee, service trees and some fruit trees and banana mats are cut down or severely pruned leaving only long cycle fruit and timber trees intact. We quantify whether available light is sufficient for food grain production during coffee and tree replanting using thresholds from studies on the quezungual system (Hellin et al., 1999) and field experience among the authors. For each of the steps and case studies we present data on density, species richness, Shannon diversity and canopy openness and the total score for income and food (**Table 3**). **Figure 6** illustrates the changes in canopy coverage and distribution through the different scenarios for the one case, while the other cases are available in **Supplementary Figures 4–6**.

Income With Habitat (Sc1)

In this first scenario we explored the contribution of increasing upper story timber trees or trees with high habitat contribution and very little light interception. The scenario is proposed given the paucity of timber trees in the great majority of the plots. Even when they are present, densities are low. At the scale of a hectare, the target was 50 additional individuals from five different species. In the typologies with low overall density and more available light the individuals can be added filling in areas with low shade level without generating excess shade, while for higher density plots, trees with lower timber potential or with lower habitat benefits or bananas were substituted. At the scale of 1,000 m^2, one individual of each species (15 year old tree for timber species and 10 year old tree for *Cecropia* which indicate the dimensions of the individual) of the following species was added or substituted into the spatial model:

– *Nectandra* spp represents several native species with well-recognized timber potential. Both flowers and edible fruits contribute to food sources for wildlife. The species have recalcitrant seed which represents a challenge to broader use which could be addressed through local and informal seed systems managed by growers who identify superior trees and notify neighbors and other contacts in moments of seed availability.

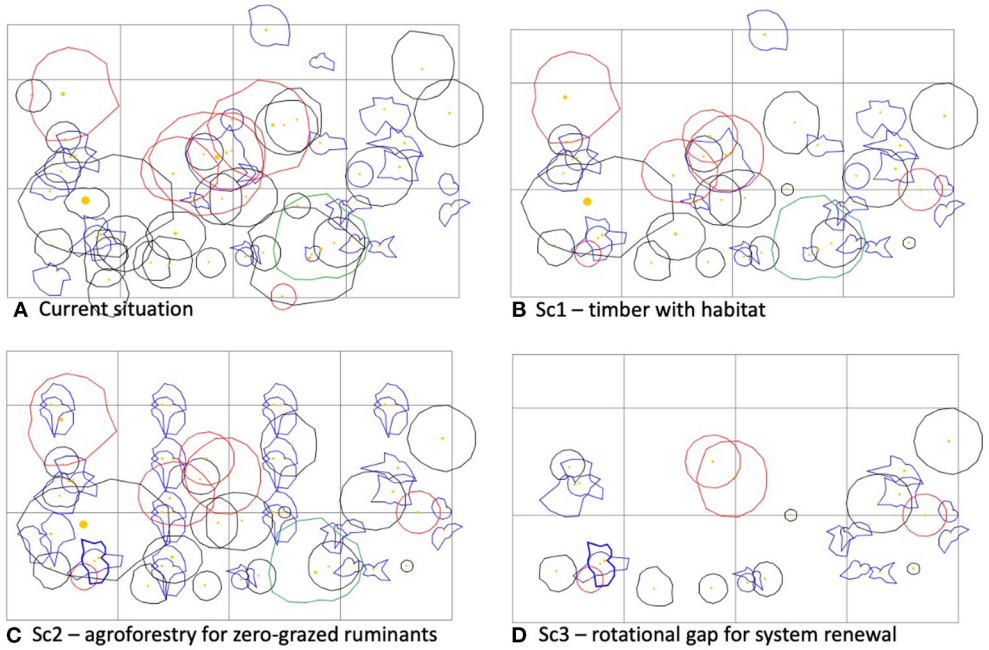

FIGURE 6 | Changes in canopy coverage and distribution for the current plot and three different scenarios for the MIX plot case. Four canopy components are graphed—banana (blue), *Inga* (green), timber trees (red), and other trees (black). **(A)** Current situation. **(B)** Sc1—timber with habitat. **(C)** Sc2—agroforestry for zero-grazed ruminants. **(D)** Sc3—rotational gap for system renewal.

- *Platymiscium parviflorum* is a legume with storable seed which is available to a limited extent through seed services. The habitat contribution is primarily during flowering.
- *Dalbergia retusa* has a very prized timber and is currently found slightly outside current coffee zone at lower altitudes and drier conditions. Climate change over the next decades suggests that growers may anticipate increasing temperatures and more erratic rainfall by planting species adapted to future conditions. The habitat contribution of *Dalbergia*, a legume, is primarily flowers for bees.
- *Cecropia* spp are highlighted for their habitat potential by Peters et al. (2016). Four species are reported for Nicaragua - *obtusifolia* from 0 to 900 m, *peltata* from 0 to 1,200 m, *silvicola* at higher elevations in a more restricted zone from 1,200 to 1,400 m and *insignis* in wetter conditions from 0 to 1,000 m. Natural regeneration of *Cecropia* may provide sufficient plants to achieve 10–20 plants/ha. Young plants would require protection from eradication during weed control practices and minimal pruning when trees are young. The high and very open canopy of *Cecropia* has little impact on light availability, while providing flowers, leaf sprouts and fruits to birds, bats and mammals throughout the year.

The addition of five individuals representing timber and habitat potential in the 1,000 m^2 plot increases timber tree density and Shannon diversity in three of four cases. Diverse tree density declines in three cases (**Table 3**). For these cases smaller or clumped trees were eliminated, often species with only one individual. The canopy openness is shifted or kept close to 50% and variability within plot is slightly improved. The benefit contribution for Income (IN) from multi-year income (MYE) increases but declines overall due to reduction in banana stem density. Habitat benefits (not shown) increased based on *Cecropia* density. The top view profile (**Figure 6**) illustrates reduced canopy overlap in the upper story which timber and habitat trees occupy.

Monthly Food and Income (Sc2)

The noticeable gap for monthly income and household consumption from current coffee agroforestry spurred us to review shade tolerant alternatives to banana. Among spices and essential oil crops, some like cardamom are currently understory associated crops, although not with monthly income potential. We concluded that leguminous fodder production might provide a basis for zero-grazing milk production in coffee zones. Rather than mid-story legume trees like *Erythrina* for fodder we propose hedge row legumes and cut-and-carry grass on field boundaries, primarily for greater labor efficiency in daily fodder harvest. Reject bananas of market cultivars or highly productive cultivars with low market potential make up the third component of the proposed diet:

- Legume hedges composed of *Leucaena* and *Calliandra* on 1 m spacings with a potential of bimonthly harvests of 2 kg/plant.
- Cut-and-carry fodder in hedges with *Pennisetum purpureum* or *Tripsacum andersonii* on 1 m spacings with a potential of bimonthly harvests of 3 kg.

– Banana at 5–6 m associated with coffee. Each mat produces 1.5 bunches per year of 20 kg. At a density of 250–350 mats per hectare managed with 1–2 tall stems/mat, banana generates around 25% shade. An estimated 100–150 mats would be needed to provide 10–15 kg of banana/day for animal consumption, but this daily ration could also be met with bunches not achieving top grade with buyers. The additional mats per hectare up to 300 may provide monthly income from banana fruit sales. Banana cultivar diversity could also be deployed for bananas as staple human diet component in substitution for potatoes, rice, cassava or even wheat flour, common staple foods purchased by coffee growing households. This diversification of cultivars either for fodder or for staple diet contribution may also allow growers more flexibility to maintain the monthly income and food function of banana even with certain disease problems of market bananas such as *Fusarium* wilt. Data in **Table 3** are reported in stems/plot rather than mats with an average of 1.25 stems per mat.

This scenario has relatively small impacts on the overhead shade for coffee fields which already had higher banana densities. Adjustments were made both to stem density/mat and mat location within the plots (**Figure 6C**). Twenty banana mats or 27 stems per plot were added in one case and banana stem density was reduced in one case from 85 to 40. Legume and grass hedges as understory may need to be oriented to more open sun locations on field boundaries, although they could also substitute for a row of coffee. Farm level biosecurity to address the spread of new diseases and food safety to limit access to specific zones of the farm may also be addressed with multi-use fodder hedges. A single milk cow fed from bananas and hedge row legume shrubs and grass could be projected to produce up to 9–10 L of milk/day and a ton of manure on dry weight basis annually. The integration of these contributions into **Table 3** highlights a major methodological challenge to the comparison of benefits in alternative scenarios and the optimization of diverse services with different units of measurement which was also highlighted by Garland et al. (2021). In the calculations in the previous section, we used individuals as a common unit to calculate total benefits, since this information was available in our data bases. In this case, the population of fodder hedges could be deployed or a factor for the conversion to milk. To partially accommodate this problem, we added understory density into **Table 3** which at the scale of the 0.1 ha plot results in an increased density of hedge row legume shrubs and clumps of grass fodder. Overall Shannon diversity and species density are also increased in three of four cases. The benefits totals for income and food are increased based on the population of fodder plants, but we could have also used milk production itself, for example days of milk production in both monthly food and income. The manure production could be mapped into coffee productivity enhancement, although our sub-categories do not currently accommodate this type of benefit. Further studies to address quantification of multiple benefits are needed.

Rotational Gap for System Rejuvenation and Reorientation (Sc3)

The brief review of the drivers of the future for smallholder multi-strata coffee production suggested that dynamic and productive coffee management with frequent pruning and replanting will be essential to respond to changing climates and diseases and availability of new varieties. Regular renovation or rapid turnover of N-fixing trees like *Inga* increases potential for biomass production and nitrogen fixation which is greater for younger trees (Nygren et al., 2012). This suggested to us that periods of high light availability or a rotational gap when coffee and service trees are uprooted or cut back should be recognized as part of the coffee agroforestry system.

Schematically for testing a rotational gap in SExI- FS, we determined the effect on light availability generated by the elimination or heavy pruning of leguminous shade trees and other trees and uprooting or thinning of banana along with uprooting or renovation pruning of coffee. We did not attempt to propose a re-oriented species composition for the new field or capture the initial phases of replanted multi-strata. Trees not eliminated are primarily timber trees which remain standing and continue to grow in the upper story of the newly planted or renovated coffee, service trees, and bananas. Fruit trees may also be conserved or renovated depending on the availability of improved germplasm and changing markets. Studies from quezungual suggest that maize should have largely open sun conditions. Isolated high canopies trees are possible with maize (farmer interviews conducted by Siles). Beans may be more appropriate in shade up to 25% shade. For the parameters in **Table 3**, the elimination of unproductive or less useful trees and the harvest of large diameter timber trees results in a decline of tree density and overall plot species which could be compensated by the additional diversity of annual crop production. **Figure 6D** shows the resulting tree canopies. Trees may also be cut back as done in quezungual. Canopy openness increases in all plots to above 75% which is enough light available for maize or beans or other annual cash crops of 3–8 months duration. If growers renovate only smaller areas of their farms in a rotational plan every year, they will continue to have income from coffee as well as annual food or income crops. Their farms will consist of multi-strata plots in different stages of development—a relatively stable component of timber trees and fruit trees with service trees kept at optimum age for N fixation or foliage or other benefits and coffee plants with high production potential. To quantify the benefit provided in terms of food, we scored multiples of 10 m^2 of planted area in annual food or income crops. In our 0.1 ha plots, only 50% is calculated as plantable with a score of 50 points of understory plants and seasonal food which can be stored (**Table 3**).

The preliminary spatial analysis of scenarios with the light capture model and system descriptors and indices of diversity and benefits provides key insights to hypothesize a conceptualized timeline that captures in greater detail a more active coffee agroforestry management approach with more targeted benefits-oriented species and stem turnover to increase system viability (**Figure 7**). The three coffee renovation cycles

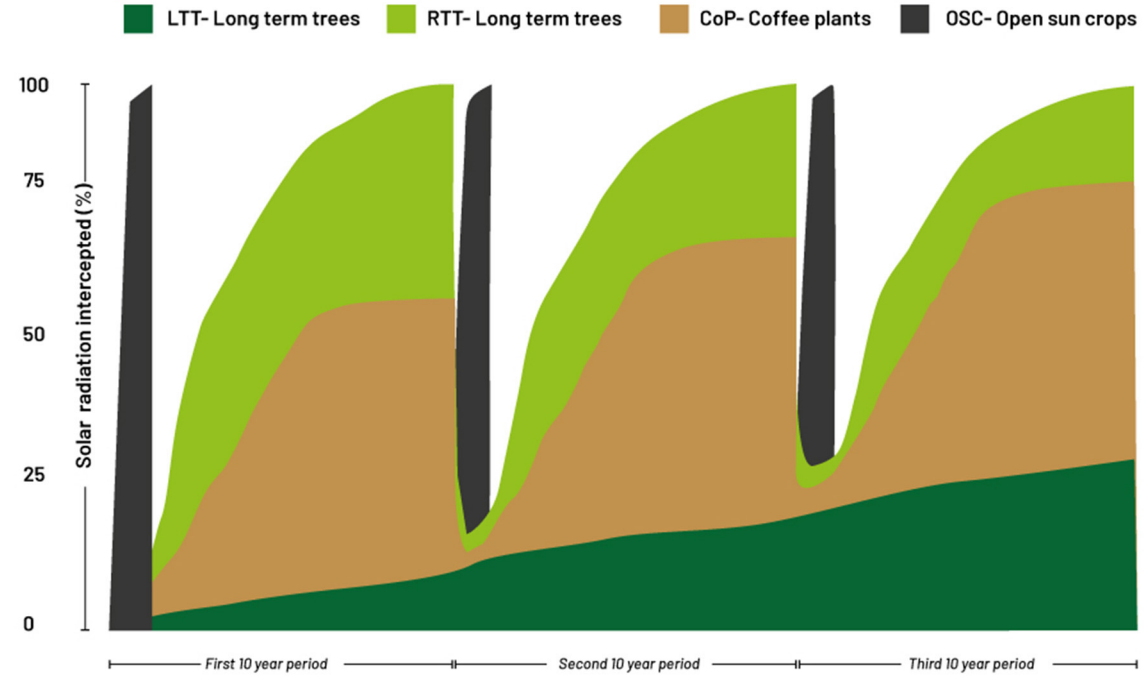

FIGURE 7 | Hypothetical dynamic shifts during three coffee renovation cycles.

shown in the timeline are punctuated by more open sun-gaps with abundant light for temporary short-term food or cash crops (Scenario 3) and a pivot point to shift coffee varieties, replant rapid turnover service trees and shift species or varieties of other associated trees, including banana (Scenario 2) while also maintaining long-term upper canopy trees (Scenario 1). Staggering the renovation cycles in different plots in the farm would distribute labor, inputs and capital demands and provide diversified income and food security across years. The timeline depicts zero biomass as a starting point and certain components return to zero at the beginning of each renovation cycle, neither of which may be the case in specific on-farm cases and would also contribute to carry-over benefits from one renovation cycle to the next beyond the long-term trees. In the discussion section that follows, we identify gaps in the literature and areas for improved data collection and analysis which address alternative pathways for more benefits-rich smallholder agroforestry.

DISCUSSION

Our summary of the current composition and diversity of coffee agroforestry fields on different continents clearly confirms the potential of high species diversity and associated local benefits in many different climatic and cultural situations to provide an enduring low-risk, resource-efficient production strategy for smallholder rural communities. The multi-strata system is recognized for conservation of soil potential, the biotic resources for complex food web relationships generating biocontrol and biodiversity habitat. Numerous studies have also shown that coffee agroforestry has useful carbon sequestration potential (e.g., Soto-Pinto et al., 2010; Pinoargote et al., 2017), although this ecosystem service may not generate much local value in and of itself. Studies which take a farm rather than a field perspective have highlighted the need to understand seasonal household wellbeing, particularly since income from coffee which is the main cash crop is highly seasonal and dependent on global factors. However, few current coffee agroforestry fields (**Table 1** and **Figure 3**) provide staple food. Income diversification appears more often to be potential rather than realized. Banana in coffee agroforestry is the exception generating both food and income throughout the year.

The conceptualized timeline in **Figure 7** maintains coffee as central to smallholder livelihoods. Not all future small growers can double or triple yields even of their current area without flooding global markets. More cost-effectives coffee productivity in a dynamic benefits-rich multi-strata may be more viable for smallholders than increased coffee productivity in monoculture. Our schematic three-step analysis (typology formation, benefits quantification using species and plot inventory, testing through spatial modeling of stem/species rearrangements testing for increased, and more targeted benefits) brought to our attention gaps in field studies and challenges in improving data collection and analysis using the local benefits framework from agroforestry as an entry point. Addressing these gaps and challenges is needed to test our hypothesis in greater detail and adjust **Figure 7** to field realities.

Among the gaps we identified:

– The focus on the tree component of standing multi-strata coffee fields should be complemented with a more complete

documentation of other species diversity including herbaceous groundcover layer and fence and hedge rows and vegetation dynamics during periods of coffee renovation when coffee agroforestry is disassembled and reassembled or converted to another use.

- A quantitative, seasonal perspective of local benefits, particularly of income, food, and habitat, is needed to orient species selection and renovation during the different phases of the multi-strata useful life. A particular challenge is the units of measure to evaluate a broad range of non-monetary benefits from wildlife and biocontrol habitat to nitrogen and recycled biomass from leguminous service trees. The units to measure food for home consumption and non-coffee income are clearly monetary, but actual measurement requires data collection methods not often deployed. We used stem density which was available in our data bases which indicates potential rather than actual benefits. The challenges of measuring non-monetary services and optimization of multiple objectives have been highlighted by other authors (Rapidel et al., 2015; Dendoncker et al., 2018).
- The multiple and changing pressures on smallholder enterprises to respond to national regulations and export market certification requirements, while also effectively combining their scare labor and capital with ecological processes, merit integrated and multi-year analysis. One-time studies do not capture the on-going strategies of smallholder farm enterprises to achieve household wellbeing with coffee production as a key activity. De Leijster et al. (2021) used time from conversion to agroforestry from 1 to 40 years in a latitudinal study of ecosystem service trajectories. While useful for environmental indicators which may link to services which increase from time of tree establishment, the data collection did not identify differing life spans for coffee, banana, service trees and timber trees, an important element in our hypothesis about the dynamic and purposeful approach needed for transformed agroforestry systems. Documentation for certification offers a potential large data base only infrequently tapped (see example—Barham and Weber, 2012) for broader analyses. However, certification requirements do not cover income diversification, food security or ecosystem services for pest control, among others, and are somewhat incipient for habitat, often using biodiversity as a general indicator.
- Studies of resource use efficiency and competitiveness of smallholder coffee have been piloted. However, the expanding tools for the study of efficiencies documented in the overview of research have not been applied to coffee agroforestry plots or farms/enterprises. Such studies can be envisioned to provide a platform for more applied indicators and tools for more forward-looking smallholders and their associations, but remain an important gap to complement the numerous studies of agroforestry diversity.

Challenges to data collection and analysis using a local benefits framework are:

- A unified analysis of very diverse benefits based on inventories of individuals and species in multi-strata systems drew our attention to the challenges in the units of measure. We used number of individuals and a simple scoring system, although clearly not all individuals have the same weight nor is the benefit linear in response to stem density. Other derived benefits such as manure did not link as easily to individual stem number. The study by Rice (2011) which contrasted fruit production and sale in two coffee zones illustrates the importance to measure actual benefits. He found that much of the fruit produced in coffee fields never reached the market and was not consumed locally.
- The seasonal breakdown of benefits contributing to household wellbeing opened a new window on understanding current systems and led us to identify potential alternatives such as zero grazing and the full sun short term crops. The seasonal breakdown is also an important perspective on species contribution to habitat. **Figure 7** was not prepared to capture the within-year seasonal dimension of coffee agroforestry, but does indicate moments for species substitution or supplementation with a seasonal filter.
- The identification and testing of scenarios guided by multiple objectives highlights the role of knowledge and measurement of responses as a basis for improved grower agroforestry management. In the scenarios applied to each plot, the initial review of the current tree by tree inventories raised many questions about each tree's contribution and possible additions and substitutions and the potential contributions of alternative species. The costs and returns of alternative multi-strata approaches can only be addressed directly based on grower experience using multi-year data. Big data analysis of coffee fields and small coffee farm enterprises could be a powerful source of recommendations on production and economic efficiency. Our data base of 140 coffee fields was useful to understand the diversity of farmer strategies, but is insufficient for expanded studies to guide management shifts.
- The spatial model to visualize alternatives does not integrate the diverse measures of efficiencies documented in an earlier section, although it did generate specific contexts to screen very different alternatives. Much more thorough characterizations of smallholder coffee agroforestry plots and farms/enterprises are needed first to move beyond just coffee production efficiency to integrate agroforestry, but also to link plot level data with household level food security, income diversification and such issues as availability, skills, and living wages of hired labor. Ho et al. (2017) in their comparison of coffee monoculture, coffee intercrops and coffee-rice found lower efficiencies in coffee-rice, but recognized possible farm household preference for reduced food security risk from coffee-rice.

These gaps and challenges on transformation alternatives of traditional coffee agroforestry lead us to visualize three future directions for a science and research agenda in support of farmer management tools:

- First, more extensive data bases are needed of small farms with coffee agroforestry as enterprises which are managing land, labor and capital to address household, market and

biotic/abiotic demands. Certification data bases may provide some data, but are incomplete. Studies of coffee agroforestry species diversity like our data bases from Nicaragua are useful, but focus only on a single moment, are missing coffee data and do not address enterprise or livelihood issues. Studies of scale, technical and allocative efficiencies cited earlier have a coffee focus and miss the contributions of the agroforestry component addressed by economies of scope. They address primarily economic cost-benefit and input-output relations. Bringing together economists with specialists on coffee and coffee agroforestry agronomy and pest management is needed to design data collection and address the different efficiencies of farms, territories and value chains. The studies on food security cited in the introduction highlight an important component of farm data collection to build a better understanding of current strategies of the households of coffee farm owners and their permanent and seasonal workers. The potential of smallholders to harness ecological intensification as an additional component of efficiencies calls for expanded indicators, some of which may are not yet well-understood like tree contribution of nitrogen and trees as habitat for beneficial organisms. The potential of coffee with agroforestry to contribute to living and prosperous incomes needs to be dimensioned by farm size, availability of markets and off-farm services and natural resource base. Waarts et al. (2019) highlight the challenges for different sub-groups of farms based on income and land size to improve livelihoods through commodity production and the vulnerabilities of very smallholders and workers. The importance of data-driven analysis is key to more effective research and policy agendas.

– The second area for science with potential to guide management tools is the knowledge base of functions of individual tree, shrub and herbaceous species and their interactions to generate regenerative potential in the coffee ecosystem. Areas needing further attention are the role of tree species and their management in coffee system nutrient cycling and microenvironment, foodweb interactions and seasonal habitat potential for different classes of fauna. The development of this knowledge base for practical application may be facilitated by defining ecosystem services bundles which are co-occurring and linked to similar management practices (Raudsepp-Hearne et al., 2010; De Leijster et al., 2021). The use of vegetation species diversity also depends on a better understanding of tree and shrub seed phenology and availability and seedling establishment parameters.
– Finally, the interface between data for certification, the demand for which is growing, and data-driven multi-strata enterprise management merits attention. We propose that relevant indicators of key system variables and simple data capture building on both ecological and enterprise understanding as well as certification demands will provide a basis for management tools. We visualize that more systematic, widespread and routine deployment of data collection linked to data storage and analysis will have local and global benefits to individual growers, their organizations, certifiers, national regulators and consumers.

CONCLUSION

Our hypothesis highlighting the opportunities for transformation alternatives for coffee agroforestry builds on the multiple benefits possible from multi-strata diversity as a production system. Smallholders have leveraged lower coffee production costs and products for household needs and diversified income from agroforestry rich with farmer knowledge and experience to participate in the highly unstable global coffee commodity market. However, challenges are multiplying—decreasing coffee margins, increasing input and labor costs, new pests and diseases, climate variability and change and increasing data demands from supply chain certification and national laws and regulations. We hypothesize that coffee agroforestry must shift from a low labor, low risk-stable return, slowly changing matrix to more active management of species and stem turnover in system renovation cycles targeted to sustaining, reorienting and intensifying ecosystem-based benefits.

The multiple benefits framework, the test using stem density by species in smallholder coffee agroforestry in Nicaragua, and the scenario building based on light availability, although primarily illustrative with many aspects to be improved, confirmed the utility of categorizing benefits in food, income, other household use, coffee productivity and habitat. Sub-categories by season, type of market and development phase of the plot (including the gap phase during system renovation) provided insights into the potential for increasing benefits with more targeted species and stem management and turnover.

The multi-cycle framework proposed in the conceptual timeline in **Figure 7** addresses the fluctuation of system components on different periods of return. Species and stem turnover generate these cycles based on the active assessment and learning by the farm household, grower association and rural community. Cultivar changes to address climate change and emerging pests, cultivars and species with greater market value, shifts to species with greater integrative functions, for example income, household food, soil building and habitat to promote biocontrol, are location- and even time-specific.

Our data bases of stem density by species of established agroforestry systems were sufficient to identify gaps in several categories of food and income benefits for coffee-growing households in northern Nicaragua which were addressed in the scenarios thereby verifying the hypothesis. The benefits ranking both of current systems and three scenarios also provided insights into data collection specifications for a more rigorous test of the hypothesis and as the basis for farm enterprise management tools. Increasing data recording by small coffee farms is currently driven largely by the demands of certification and national labor, health and environmental laws. More systematic documentation of costs and the income and food provided by agroforestry components as well as coffee follows already available farm management procedures. The methods for the quantification of agroforestry contribution to ecological processes based on species and tree size are still incipient primarily for academic use and not applied to farm management. The diverse efficiency analyses used by economists provide a starting point for more exhaustive hypothesis

testing on agroforestry benefits by farm size, proportions of labor, inputs and land, degree of diversity and response capacity to extreme market and climatic events. Integration of ecological processes into the efficiency analysis is essential requiring collaboration among farm management economists, agronomists, IPM specialists and ecologists. In our vision these more academic studies can and should lead to more useful applied farm management tools and data-driven alternatives for greater ecological, economic and livelihood viability for farm households and grower associations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CC identified the call for article and proposed a article using existing data sets. CS proposed a article on hypothesis and theory to explore the larger context of coffee agroforestry, developed initial arguments from the literature, developed the scoring system, and contributed to the interpretation of the results. PS and CC carried out the field data collection. PS did statistical analysis. CC and CS took the lead in writing the manuscript. All authors provided critical feedback, helped shape the research, analysis, and manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2022.808207/full#supplementary-material

Supplementary Table 1 | Species by attributed local benefits provided. PE, Coffee productivity enhancement; IN, Income; FO, Food; HO, other household use; HA, Habitat for biodiversity.

Supplementary Figure 1 | Species accumulation curves for three data sets and four typologies.

Supplementary Figure 2 | Shannon index distribution for 140 plots in three data sets and four typologies.

Supplementary Figure 3 | Species density distribution for 140 plots in three data sets and four typologies.

Supplementary Figure 4 | Changes in canopy coverage and distribution for the original plot and three different scenarios for a Musa-dominated plot (MD). Four canopy components are graphed—banana (blue), Inga (green), timber trees (red), and other trees (black).

Supplementary Figure 5 | Changes in canopy coverage and distribution for the original plot and three different scenarios for an *Inga*-dominated plot (ID). Four canopy components are graphed—banana (blue), Inga (green), timber trees (red), and other trees (black).

Supplementary Figure 6 | Changes in canopy coverage and distribution for the original plot and three different scenarios for a plot dominated by other trees (TD). Four canopy components are graphed—banana (blue), Inga (green), timber trees (red), and other trees (black).

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Shaded-Coffee: A Nature-Based Strategy for Coffee Production Under Climate Change? A Review

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Coffee is deemed to be a high-risk crop in light of upcoming climate changes. Agroforestry practices have been proposed as a nature-based strategy for coffee farmers to mitigate and adapt to future climates. However, with agroforestry systems comes shade, a highly contentious factor for coffee production in terms of potential yield reduction, as well as additional management needs and interactions between shade trees and pest and disease. In this review, we summarize recent research relating to the effects of shade on (i) farmers' use and perceptions, (ii) the coffee microenvironment, (iii) pest and disease incidence, (iv) carbon assimilation and phenology of coffee plants, (v) coffee quality attributes (evaluated by coffee bean size, biochemical compounds, and cup quality tests), (vi) breeding of new Arabica coffee F1 hybrids and Robusta clones for future agroforestry systems, and (vii) coffee production under climate change. Through this work, we begin to decipher whether shaded systems are a feasible strategy to improve the coffee crop sustainability in anticipation of challenging climate conditions. Further research is proposed for developing new coffee varieties adapted to agroforestry systems (exhibiting traits suitable for climate stressors), refining extension tools by selecting locally-adapted shade trees species and developing policy and economic incentives enabling the adoption of sustainable agroforestry practices.

Keywords: agroforestry, bean quality, climate change, *Coffea*, crop management, hybrid, microclimate, shade

INTRODUCTION

The coffee sector generates a global annual income exceeding $200 billion with a tendency for steady market growth of 2.2% every year (The Coffee Guide, 2021). There are more than 12.5 million coffee farms at the beginning of the coffee value chain, with *ca*. 60% belonging to smallholders, producing coffee on <5 ha (Enveritas, 2019). A large proportion of smallholder coffee farmers are living below the poverty line of $3.20 USD a day (The Coffee Guide, 2021).

As for several other economically important crops, ecological modeling approaches have predicted that coffee production will be threatened in the forthcoming years due to climate change (Chengappa et al., 2017; Asayehegn et al., 2018; Rahn et al., 2018; Grüter et al., 2022). The forecasted weather variation includes perturbations in intra- and inter-annual rainfall patterns, more frequent drought periods, elevated temperatures, as well as a shift in geographical coffee growing regions (Bunn et al., 2015a; de Sousa et al., 2019). In fact, the annual world coffee supply is already subjected to instability, mainly triggered by recurrent periods of drought accompanied by high temperature and irradiance as well as by episodes of frost (Caramori et al., 1996; Morais et al., 2006; Rigal et al., 2020a; Braga et al., 2021) affecting plant development, flowering, fruit set, and production (DaMatta and Ramalho, 2006). These impacts are believed to mainly affect *Coffea arabica* (Arabica) due to an inherent sensitivity to elevated temperatures, drought, pest, and diseases. *Coffea canephora* has previously been predicted to respond better to climate change than Arabica (Jayakumar et al., 2017; DaMatta et al., 2018). However, recent studies suggest that the alleged "heat tolerance" boasted by *C. canephora* (Robusta) plants could have been overestimated (Kath et al., 2020). Others point to elite Arabica genotypes which exhibit a heat and drought tolerance when simulated under climate change scenarios such as elevated air CO_2 (eCO_2) (Martins et al., 2014; Rodrigues et al., 2016; Ramalho et al., 2018; DaMatta et al., 2019; Avila et al., 2020a,b; Semedo et al., 2021). Still, impacts on both Arabica and Robusta plants are expected to occur, thus on-farm climate change mitigation and adaptation practices will be essential for a sustainable coffee management and productivity.

The cultivation of coffee under shaded agroforestry systems (AFS) (**Figure 1**) or intercropped with other trees/crops are among the agricultural practices able to improve microclimate conditions and mitigate the negative effects of climate change to coffee plants (Jaramillo et al., 2013; Partelli et al., 2014; Oliosi et al., 2016; Pham et al., 2019). Traditional coffee cultivation practices are often carried out by poorer farmers (Jha et al., 2014). These practices consist of growing coffee under native forest trees with a layered canopy. Generally, the higher the coffee cultivation intensity, the lower the layer complexity and plant diversity becomes. In its most intensified form (full-sun mono-crop systems), all plant diversity is lost. Since the late 90s, coffee farmers have moved toward intensive production systems, increasing inputs and decreasing the area of traditional shade-grown coffee. In new coffee growing regions (i.e., Southeast Asia), full-sun (FS) coffee cultivation systems dominate other forms (Jha et al., 2014), as is the case in Brazil, the world's largest coffee producer.

AFS enables several positive impacts such as buffering extreme temperatures and minimizing water loss by reducing soil evaporation and crop transpiration (DaMatta, 2004; Lin et al., 2008; Jha et al., 2014; Gomes et al., 2020; de Carvalho et al., 2021) as well as contributing to on-farm carbon sequestration (Ehrenbergerová et al., 2016). For this reason, AFS is today considered a nature-based solution for perennial crops which are sensitive to climate change. AFS has been shown to also reduce pests and diseases, e.g., by bolstering *in-situ* natural enemies of pests (Ratnadass et al., 2012). However, controversy surrounds the use of shade trees in coffee production due to their negative impacts on coffee growth and yield, as well as their potential to exacerbating biotic stressors (especially foliar disease such as coffee leaf rust—CLR) (Haggar et al., 2011; Avelino et al., 2020; Durand-Bessart et al., 2020; Gichuru et al., 2021).

The implications of AFS on coffee cup quality are also of the upmost importance, and the demand for specialty, high quality, and sustainably sourced coffee has skyrocketed over the last decade for both Arabica and Robusta coffee (van der Vossen et al., 2015). Industry and consumer demands call for a challenging feat—coffee cultivars/clones capable of coping with climate change combined with high yield and cup quality, produced under sustainable agricultural practices.

Coffee-AFS are living systems, highly variable to their given environments. A healthy coffee AFS should integrate localized, on-farm management practices including regulation of soil, water, tree, pest and disease to ensure ecological, economic, and social benefit of the system (Sebuliba et al., 2021). Given the documented variation within coffee-AFS, it is of the upmost importance for the coffee community to stay informed of the newest findings relating the interactions between shade and the coffee crop. For this reason, this literature review aims to bring together recent findings from studies (mainly performed in field trials) relating to the use of agroforestry compared to FS (open cultivated) systems in Arabica and Robusta coffee production. We evaluate recent work(s) in light of several important aspects associated with coffee management and future sustainability. This includes the effects of shade on (i) farmers' use and perceptions, (ii) the coffee microenvironment, (iii) pest and disease incidence, (iv) carbon assimilation and phenology of coffee plants, (v) coffee quality attributes, (vi) breeding of new Arabica coffee F1 hybrids for future AFS systems, and (vii) coffee production under climate change. The interactions between these key aspects can influence whether coffee farmers adopt the use of shade trees (**Figure 2**). Through this review, we begin to decipher whether shaded systems are a feasible, nature-based strategy for coffee farmers faced with climatic hazards through exploration of these key aspects.

ANALYSIS OF THE BIBLIOGRAPHY

In this work, we reviewed literature concerning coffee production under controlled field studies using shaded environments or within agroforestry-like systems. We based the review on

Abbreviations: AFS, agroforestry systems; CLR, coffee leaf rust; eCO_2, elevated air CO_2; FS, full-sun; WoS, web of science.

FIGURE 1 | Different ways of cultivating coffee under shade. **(A)** Terraced Arabica coffee on an elevated site in amongst a mixture of trees species. **(B)** Experimental trial with *C. canephora* (Conilon cv.) in an agroforestry system intercropped with a single shade tree species. Photos © Benoit Bertrand and © Pierre Marraccini.

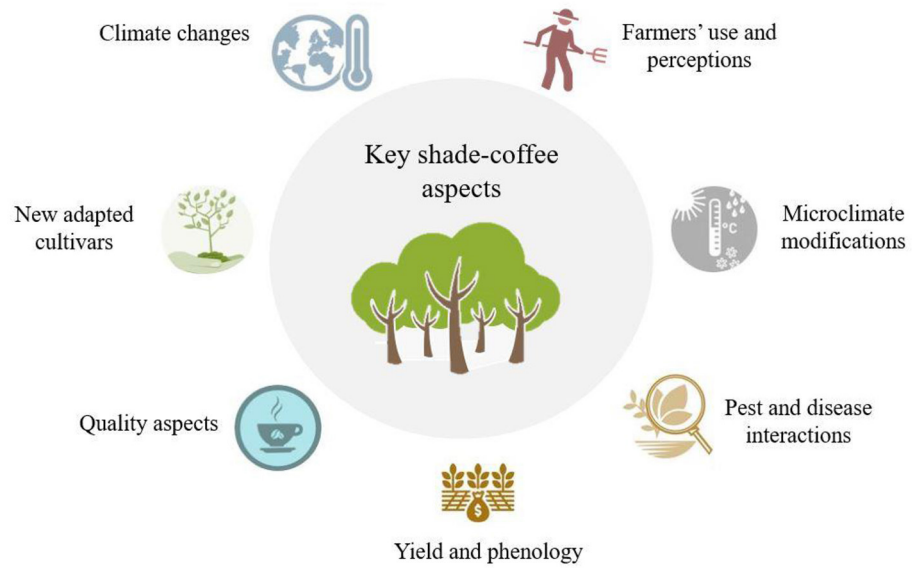

FIGURE 2 | Key aspects relating to shaded-coffee systems. Any of the given factors may bear weight in the on-farm decision-making relating to the use and management of shaded-coffee systems.

database searches in Web of Science (WoS) and Scopus, as well as supplementary publications retrieved through other means (publication alerts, sent from colleagues etc.).

The core keywords used in the database searches were "agroforestry" AND "coffee" AND "climate." This allowed for the inclusion of studies relating to both the *C. arabica* L. and *C. canephora* Pierre ex A. Froehner species. Additional key words were used in combination with the core keywords to cover each thematic area. These included (i) "farmer" AND "perception" OR "farmer" AND "use," (ii) "microclimate" OR "aboveground," (iii) "pest" OR "disease," (iv) "phenology" OR "carbon" AND "assimilation," (v) "quality" OR "biochemical" OR "organoleptic," (vi) "hybrid," and (vii) "climate change." The date range was open for each search conducted and thus results spanned from 1976 to 2022 (including pre-prints). However, the majority of the studies found were dated between 2010 and 2022. Seven separate searches were conducted for each thematic area via WoS and Scopus on the 18th January 2022. In total 246 publications were reviewed across all thematic areas. The majority of the shade-related coffee research was found to be located in AFS-promoting countries such as in Latin America (e.g., Costa Rica, Colombia, Mexico, and Nicaragua). Given this, a geography bias was detected, with a large number of the work reviewed coming from these countries. This is likely connected to the long coffee cultivation history in Latin America and the rather recent commercial expansion into South East Asian countries (i.e., Vietnam) and Africa (i.e., Kenya, Tanzania, and Uganda). Despite this bias, many large coffee producing countries (e.g., Brazil, India, Kenya, Uganda, and Vietnam) are beginning to integrate AFS as a management approach. Therefore, some studies from

these geographies were found (but to a lesser extent than the Latin American studies).

COFFEE FARMERS' USE AND PERCEPTION OF SHADE TREES

There are no global data sets pertaining to the extent or share of coffee AFS compared to mono-crop or FS coffee to date. There is however, scientific consensus that coffee AFS is gradually diminishing in its share of total coffee area and is under threat from policies favoring intensive, highly productive systems (Jha et al., 2014; Albers et al., 2021; Harvey et al., 2021). According to the most recent attempt to quantify the share of AFS in coffee producing regions, Jha et al. (2014) found that <24% of the coffee area worldwide in 2010 was managed with traditional and diverse shade, 35% with sparse shade and 41% under FS. This study followed up on a 1996-study that reported massive conversions of shade coffee to intensively managed sun systems during the period from 1970 to 1990 in Latin America (Perfecto et al., 1996). This trend has continued until today, though at lower rates than previously recorded (Harvey et al., 2021). During the 1990's, coffee areas in Latin America and Africa have declined, while areas in Asia have increased, most notably in Vietnam, under intensive FS systems and thereby exacerbating the trend toward a lower share of shaded coffee (Jha et al., 2014). Key drivers to this trend were the national policies which targeted increasing yields to support greater exports. Breeding efforts have also traditionally focused on plant vigor and productivity (van der Vossen et al., 2015). However, the recent implementation of new agricultural policies, both public and private, and breeding efforts turned their attention to shaded coffee and intercropping systems, often as an adaptation strategy to climate change or as part of a certification process (Rice, 2018; Vanderhaegen et al., 2018; Bertrand et al., 2021). Recent efforts, notably public programs in Vietnam and China, have pushed for the conversion of large areas of full sun coffee into shaded systems (Rigal et al., 2018) and for the introduction of fruit trees intercropped with coffee trees (Thuy et al., 2019).

Coffee AFS vary in size and complexity, ranging from simple mixtures involving a few shade tree species planted at regular intervals within the rows of coffee trees to traditional or so-called "rustic systems," where coffee is planted in plots of forests. In a rustic coffee AFS, numerous tree species can provide an almost complete shade cover through a multi-strata tree canopy (Somarriba et al., 2004). Shade tree abundance and diversity varies according to a number of farm and farmer characteristics, such as farmers' livelihood and diversification level, available space on the farm, and availability of workforce labor (Méndez et al., 2009; Rice, 2011; Lamond et al., 2016; Robbins et al., 2021).

The coffee crop under AFS is often less intensively managed than in FS systems, with fewer inputs in form of labor and expensive agrochemicals going into the production. Pruning of shade trees and leaf litter will reduce, to some extent, the need for chemical fertilizers (Bravo-Monroy et al., 2016). Additionally, the multi-strata agroforestry systems may reduce occurrences of certain pests and diseases (Soto-Pinto et al., 2002; Durand-Bessart et al., 2020) and thus the need for pesticide application. However, coffee AFS are not always the panacea for pest control, and these complex biotic networks were also reported to favor specific pests and pathogens depending on environmental factors (Allinne et al., 2016).

AFS use is common amongst small-scale coffee farmers, as labor is mainly family-based and used to manage all types of crops in the AFS of limited size. This is also associated with little access to agrochemical inputs (either due to market constraints and/or high costs), altogether resulting in lower management costs per hectare (Jezeer et al., 2017). On larger farms, labor may become the limiting factor for diverse shade systems, as found by Robbins et al. (2021) in India, where larger farms had higher abundance and diversity of shade trees than small farms due to better availability of hired labor. In a review of studies spanning 26 years across Latin America, Jezeer et al. (2017) reported that lower costs and supplementary income from shade tree products made shaded systems more profitable than non-shaded systems. Products from shade trees, such as fruits, firewood, timber and other materials, can make up a substantial part of the total income from coffee AFS (Rice, 2008; Souza et al., 2010; Thuy et al., 2019), and highly diverse coffee AFS increase the household's food and nutrition sources, especially in the shortage season (Jemal et al., 2021). Payment for ecosystem services can also generate additional source of revenues for AFS-coffee farmers (Cole, 2010; Thuy et al., 2021). Diversification of income from shade trees can be a safety net or gap-closing strategy especially important during times of low coffee prices (Gordon et al., 2007; Rice, 2011).

AFS must be locally tailored to answer farmers' constraints and needs, and to cope with local environmental conditions. The criteria established by each farmer to select shade trees are undoubtedly useful to other farmers, but one farm system can not necessarily be replicated to another site (Souza et al., 2010). For instance, the adequate degree of shade varies according to the elevation (Rahn et al., 2018). Coffee farmers in Kenya perceived dense shade as potentially problematic at high elevations, while they considered it beneficial at low elevations in regulating temperature, sun damages and pest incidences (Lamond et al., 2016). This may change in future scenarios with climate change and elevated atmospheric CO_2, where 50% shade at high elevations will become beneficial as found in yield models by Rahn et al. (2018). In Uganda, coffee farmers selected perennial shade trees that were fast growing, with small leaves and wide crowns (Sebuliba et al., 2021). This tendency often results in the selection of exotic species over native trees (Graham et al., 2021), which no doubt alters local biodiversity and conservation aspects. Incompatible shade trees (having negative impacts on coffee) were often tolerated if they provided other benefits/services to the farming household (Graham et al., 2021). In their study in Brazil, Souza et al. (2010) found that farmers' first criterion for selecting tree species was compatibility with coffee, i.e., trees with deep roots and that would not bring sanitary problems to coffee trees, followed by biomass production, labor needed for tree management, and income diversification. In that study, shade itself was not a criterion for shade tree selection. Another study in southwest China found that farmers preferred trees with dense canopies and high economic returns despite their negative

impact on coffee yield (Rigal et al., 2018). By contrast, a study in Indonesia found that coffee farmers selected tree species as a means of increasing coffee yield (Zaitunah and Ahmad, 2021). In Ethiopia, the decision to adopt AFS was found to be first determined by the provision of direct economic benefits and, to a lesser degree, by social factors such as gender, family size, educational level, and land tenure (Gebru et al., 2019). A study in Mexico found that farmers chose 40–80% shade cover to mitigate climate shocks, such as long heatwaves (Ruiz-García et al., 2021), and that the indigenous Huastec Mayan farming communities harbored a high number of edible plants in their coffee AFS (Heindorf et al., 2021). It is therefore essential to adapt AFS locally to answer farmers' needs in favor of the adoption and scaling up of shaded-coffee systems and must also fit with farmers' perceptions of shade trees. Nevertheless, providing information regarding shade tree benefits to farmers with limited experience with coffee AFS management is also important. Farmers near Mt. Elgon in Uganda, who had received training as part of a Trees of Food Security program, had a more positive view on the management costs and the benefits from shade trees compared to non-participating farmers (Buyinza et al., 2022).

Several studies have shown that farmers are aware of the trade-offs involved in introduction of shade tree species in coffee farms, and that they understand the impacts of agroforestry practices on coffee phenology and productivity (Cerdán et al., 2012; van der Wolf et al., 2016; Rigal et al., 2018; Dumont et al., 2019). Some of these studies even reveal in-depth knowledge of the complex causality between shade trees and agronomic services, such as soil conservation, nutrient cycling or pest, and disease management, as well as adverse competitive effects between plant species (Bagyaraj et al., 2015; Lamond et al., 2016; Liebig et al., 2016; van der Wolf et al., 2016; Nesper et al., 2017, 2018; Dumont et al., 2019). This extensive knowledge and its impact on farming practices is reflected in the farmers management of the percentage of shade throughout the year to regulate humidity, rainfall interception, light interception, and fungal diseases, such as CLR and American leaf spot (Cerdán et al., 2012). Farmers' willingness and ability to convert to and manage coffee AFS will depend on the economic performances of these systems and on the implementation of financial mechanisms to reward the adoption of more sustainable practices and the production of higher quality coffee (Borrella et al., 2015; Verburg et al., 2019). This transition will also require farmers' access to financial credit, as well as access to information and to adequate seedlings of both shade trees (Rigal et al., 2018), and coffee (van der Vossen et al., 2015). Furthermore, the adoption of shaded systems to coffee crops must be accompanied by the design of suitable AFS and their associated management practices. In traditional coffee growing areas, farmers' extensive local ecological knowledge can therefore be an asset in designing and scaling up locally tailored agroforestry practices (van der Wolf et al., 2016).

SHADE AND THE COFFEE MICROENVIRONMENT

Shade trees can be beneficial in environments that are becoming increasingly less suitable for coffee cultivation (Ehrenbergerová et al., 2021). The presence of shade trees in coffee farming systems has generally been associated with favorable microclimate modifications such as lower air temperature fluctuations, increased air relative humidity, lower wind speed (see **Table 1**) and decreased frost damages (**Figure 3**). Research concerning ecosystem services by shade trees in coffee plantations have also noted an overall positive impact on soil fertility, total organic matter, and nutrient cycling, a reduced soil evaporation and soil erosion, as well as higher on-farm sequestration of carbon (Cannavo et al., 2011; Dubberstein et al., 2018; Guillemot et al., 2018; Padovan et al., 2018; De Giusti et al., 2019; Sarmiento-Soler et al., 2019; Jácome et al., 2020; Villarreyna et al., 2020; Zaro et al., 2020). An aspect relating to the belowground competition is weed control, with a significant reduction of weeds present under shaded coffee systems compared to full sun (Nestel and Altieri, 1992). Moreover, the weed species' commonly found in shaded systems (such as *Commelinaceae* spp.) tend to be less competitive with coffee plant for resources than those more prominently found in FS plantations such as *Poaceae* spp. and *Compositae* spp. (Staver et al., 2001).

As a general rule, the selection and pruning of shade trees should favor the aboveground positive impacts while reducing the competition between coffee and shade trees for light, water and nutrients (Beer et al., 1998; Souza et al., 2010; van der Wolf et al., 2016). The selection of shade tree species with deeper root systems is especially important to ensure belowground complementarity rather than competition (Padovan et al., 2015; Rigal et al., 2020a). Here, we focus the review on the impact of shade trees on aboveground growing conditions, which will be directly impacted by climate change.

Temperatures

Changes in temperatures and intra- and inter-annual rainfall patterns will negatively impact the suitability of large areas traditionally suitable for coffee production (Bunn et al., 2015a; Semedo et al., 2018; de Sousa et al., 2019; Gomes et al., 2020). Although some areas might benefit from new climatic conditions (especially areas at high elevations which will see an increase in temperatures and a shift from sub-optimal to optimal conditions) (Ceballos-Sierra and Dall'Erba, 2021) overall coffee production is expected to decline due to global warming (Kath et al., 2020). In addition, climate change is expected to increase the frequency and severity of extreme temperature events, both for heatwaves or cold spells, which will further impact coffee production. Shade trees offer a mitigation strategy for these climatic hazards, with overall cooler daytime air temperatures, thus contributing to maintain suitable growing conditions at lower elevations (de Souza et al., 2012; Rahn et al., 2018; Gomes et al., 2020). A buffering impact of shade trees on air temperatures has been reported in numerous studies, with minimum night temperatures found to be 0.5–2°C higher than under FS, and maximum daytime temperatures 4–5°C lower compared to FS (Lin, 2007; Siles et al., 2010; Rigal et al., 2020a; Merle et al., 2022). This buffering impact offers the double asset of protecting coffee trees from climatic hazards such as frost (**Figure 3**) and lowering heat stress during peak temperatures in the dry season, therefore providing conditions more suitable for photosynthetic activity (van Kanten and Vaast, 2006).

TABLE 1 | Summary of microclimate factors influenced by shade in the coffee field.

Microclimate factor	Condition	Effect	References
Daily temperature fluctuations	Natural shade trees	Reduced	Staver et al., 2001 DaMatta and Ramalho, 2006
Maximum daily temperature	Natural shade trees (incl. *Cajanus cajan* and *Toona ciliata*)	Reduced by 3–5 °C	Staver et al., 2001 Morais et al., 2006 Oliosi et al., 2016
Average daily leaf temperatures	Sub-optimal (>700 m.a.s.l.)	Reduced by 4 °C	Vaast et al., 2005
	Optimal conditions (>1,100 m.a.s.l.)	Reduced by 2 °C	Vaast et al., 2005
Relative air humidity	Natural shade trees and artificial shade (during the dry period)	Increased	DaMatta and Ramalho, 2006 Oliosi et al., 2016 Coltri et al., 2019
Wind speed	Natural shade trees and artificial shade (during the dry period)	Reduced by 22–99%	DaMatta and Ramalho, 2006 Coltri et al., 2019
Radiation	Natural shade trees and artificial shade (during the dry period)	Reduced by 15–90%	Morais et al., 2006 Coltri et al., 2019
Coffee leaf transpiration (per unit leaf area)*	Natural shade trees (*Eucalyptus deglupta*, *Terminalia ivorensis,* and *Erythrina poeppigiana*)	Reduced	van Kanten and Vaast, 2006
Frost damage protection	Natural shade trees (*Cajanus cajan, Bischofia javanica, Cinnamomum camphora,* and *Jacaranda mimosifolia*)	Increased	Morais et al., 2006 Rigal et al., 2020b
Soil organic matter	Natural shade trees	Increased by 10%	Rigal et al., 2020a
Soil microbial abundance (bacterial and fungal communities including arbuscular mycorrhiza)	Natural shade trees Natural shade trees	Increased by 64% Enhanced soil microbial fauna	Rigal et al., 2020a; Bagyaraj et al., 2015
Soil enzymes (involved in C and N cycling)	Natural shade trees	Increased	Rigal et al., 2020a

**However the combined transpiration of shade trees and coffee plants contributed a larger overall eco-system transpiration, thus reducing the overall water availability compared to full sun conditions (van Kanten and Vaast, 2006).*

FIGURE 3 | Frost protection of coffee plants by agroforestry practices. Young *C. arabica* plants (16 months old) in an experimental field of the Northern Mountainous Agriculture and Forestry Science (NOMAFSI) station of Mai Son (Son La province, Vietnam) under either agroforestry (AFS) with *Leucaena leucocephala* or full sun (FS) conditions and affected by frost (10th December 2019). AFS coffee plants were not damaged by frost (shown with green leaves). FS coffee plants damaged by frost are with brown and inclined leaves after a couple of hours and died within a day. **(A)** Photograph taken in the AFS trial showing the FS trial. **(B)** Photograph taken in the FS trial showing the AFS trial. Note: the smoke seen in photograph **(B)** was the result of controlled fires, which were lit the day after the first frost event. This was a short-term management practice intended to warm the coffee field microenvironment above 0 °C in subsequent nights. Photos © Philippe Vaast.

Water-Use

Coffee was found to transpire more per unit leaf area in full sun than under shade, which indicates a higher level of environmental stress in non-shaded conditions, due to higher irradiance, wind speed, air temperature and vapor pressure deficit (VPD) in FS environment than in shaded conditions (van Kanten and Vaast, 2006; Lin, 2010; Coltri et al., 2019). Lower water use (due to reduced transpiration rates) could potentially become an important feature under future conditions of limited water availability namely due to more frequent and extended periods of drought (accompanied with greater high air temperatures) (DaMatta et al., 2018; Sarmiento-Soler et al., 2019; Byrareddy et al., 2021). However, the combined transpiration of shade trees and shaded coffee plants may contribute to a larger ecosystem transpiration volume, with an overall reduction of water availability under shade compared to full sun (Sarmiento-Soler et al., 2019). This is especially relevant for coffee production areas where an extension of the dry season period is forecasted due to climate change (Cannavo et al., 2011). However, recent attempts to compare water loss between coffee-AFS and FS by a system of in-field sensors and pluviometers showed an overall higher water loss in unshaded environments (de Carvalho et al., 2021). The detrimental impact of competition for water by shade trees in AFS has been recently reported in cocoa systems (Abdulai et al., 2018). This study showed a direct link to cocoa tree mortality in the shaded systems, when facing extreme drought conditions, highlighting the importance of carefully selecting shade tree species capable of extracting water from deeper soil horizons compared to the accompanying crop plant/tree (Bayala and Prieto, 2020; Muñoz-Villers et al., 2020).

PEST AND DISEASE INCIDENCE UNDER SHADE

The shaded-coffee environment within AFS hosts biological richness in terms of tree species, epiphytes, mammals, birds, reptiles, amphibians, and arthropods (Perfecto et al., 1996, 2005; Moguel and Toledo, 1999; Jezeer et al., 2019; Udawatta et al., 2021). Coffee-AFS provide ecological services, which in turn might benefit coffee production, for example by lowering dominance of pests through both direct and indirect competition (Kellermann et al., 2008; Perfecto et al., 2014). Moreover, the coffee plants themselves naturally attract a large range of natural enemies to pests and diseases, including lizards, ants, lady beetles, mites, predatory, and parasitoid wasps, and microorganisms such as entomopathogenic fungi (Perfecto et al., 2021; Venzon, 2021). Monoculture farming of coffee however, does not provide the adequate environment nor nutrition to maintain a high level of these natural enemies in the field.

The ecological interactions in coffee agroecosystems relating to prominent pests and diseases such as the coffee berry borer beetle (*Hypothenemus hampei* Ferrari), CLR (caused by the fungus *Hemileia vastatrix* Berk. & Broome), or the American leaf spot disease (caused by the fungus *Mycena citricolor* Berkeley & Curtis) are still being elucidated today (Avelino et al., 2020; Castillo et al., 2020; Cerda et al., 2020; Granados-Montero et al., 2020; Hajian-Forooshani et al., 2020; Merle et al., 2020).

Results reported on the incidence of pests and diseases vary according to site conditions (altitude etc.), farm management, shade tree species, density of shade, and as well as the presence of forest areas on and/or surrounding the farm (Karp et al., 2013). In fact, some studies showed a higher incidence of pest or disease infestation under shade vs. FS conditions (Soto-Pinto et al., 2002; Bosselmann et al., 2009; Avelino et al., 2012, 2020; Bukomeko et al., 2018; Durand-Bessart et al., 2020). By contrast, other reports showed pest and disease reduction in coffee under AFS due to the presence of more birds and ants, as well as microclimate modifications in the shade compared to the sun fields (Johnson et al., 2010).

Specific shade tree traits such as canopy openness and leaf area have been recently found to explain most microclimate conditions (Merle et al., 2022), and significantly relate to CLR incidence levels (Gagliardi et al., 2021). These findings may serve as a "missing link" to explain the AFS-CLR dynamics and shows the importance of shade tree selection in a coffee-AFS due to potential synergistic or antagonistic effects on these cropping systems. The *Inga* sp. is a common shade tree species in coffee-AFS and has been linked to an enhanced natural pest control for the coffee plant in proximity to its canopy due to its extra floral nectars (Rezende et al., 2014). Large and heavily shaded canopies of sap-exuding shade trees have been shown to have a dampening effect on the devastating Black Coffee Twig Borer (BCTB causal beetle: *Xylosandrus compactus*) (Bukomeko et al., 2018). However, this effect is lost under the shade tree *Albizia chinensis* (Wu, 2016; Bukomeko et al., 2018). A so-called "shelter effect" of rubber shade trees was recently revealed to allow for the persistence of Coffee Leaf Miner (CLM) on coffee leaves during the cold autumn and winter seasons (Righi et al., 2013). Although a higher number of leaves were mined in the shade, overall damage to the coffee plant by CLM was lower under the shade. These studies highlight the significant impacts of pests and diseases on coffee production and in turn on farmers' livelihood, and the importance of shade tree selection for their synergistic or antagonistic effects on key coffee pests or pathogens. Further research is needed to elucidate the interactions between pest and/or disease and coffee-shaded systems, especially along varied shade, temperature, and altitude gradients for better understanding and prediction of future outbreaks (Liebig et al., 2019).

SHADE EFFECTS ON CARBON ASSIMILATION AND PHENOLOGY OF COFFEE PLANTS

Although the shade-modified microclimate may be overall favorable for the coffee plant, the associated reduction in solar irradiation in AFS is a cause of concern for many coffee-growers. Low light regime is the feature of shade-grown coffee most

commonly associated with yield reductions, and a number of works corroborate this claim (Clemens and Zablah, 1993; Campanha et al., 2004; DaMatta, 2004). Due to the lack of a common "shade metric," uncertainty lies in the levels of shade stated in the primary literature sources of the present review. Some researchers described shade in terms of a percentage of light interception at the coffee plant level (Bosselmann et al., 2009; Steiman et al., 2011; Partelli et al., 2014; Charbonnier et al., 2017). This indicator best characterizes homogeneous shade covers. Others report shade density in terms of the number of shade trees per hectare or distance from shade trees (Carvalho et al., 1961; Hernández Guerra, 1995; Baggio et al., 1997; Pilati, 2005; Siles et al., 2010; Virginio Filho et al., 2015; Araújo et al., 2016; Javier Lopez-Garcia et al., 2016), or in terms of shade tree leaf area index (Charbonnier et al., 2013; Rigal et al., 2020a), which might better describe AFS based on sparse shade trees or on fruit trees with low and thick canopy covers. A few studies were found to measure and/or simulate the level of shade cast by trees throughout the day (Charbonnier et al., 2013; Coltri et al., 2019). These approaches allowed for estimation of the amount of the radiation reaching coffee trees at different hours.

Natural shade trees provide an anisotropic delivery of light to their understory in terms of quantity and quality (e.g., assessed by the quantum ratio of red to far-red light; Vanderbilt and Grant, 1985; Chazdon et al., 1996; Lee et al., 1996). In tropical rainforests the average intensity of radiation on the forest floor ranges from 5 to 25 μmol photons $m^{-2} \cdot s^{-1}$ (within the λ 400–700 nm range), which is equivalent to around 1–3% of sunlight delivered above the canopy (Chazdon et al., 1996; Lee et al., 1996). Spectral distributions of light within naturally shaded systems can also vary significantly compared to FS conditions, because leaves from the higher canopy strata will differentially absorb more red (R) and blue (B) light and reflect or transmit more far red (FR) light. This leads to altered light quality, with a low R:FR light conditions in the lower strata (where coffee will be growing), particularly in dense canopies. This may also have implications in plant resilience/response to other environmental biotic and abiotic stresses (Courbier and Pierik, 2019). The changed R:FR ratio may also trigger morphological and anatomical changes in the coffee crop. This is frequently observed by the development of shade leaves, which are thin but have larger area than leaves developed under FS. Shade trees within a coffee AFS will therefore provide a varying spectral quantity and quality over the course of the day/season due to sun orientation, over the course of their development and dependent on which shade-tree species are present (Vanderbilt and Grant, 1985). This temporal shading effect of natural shade trees may allow for light environments that reduce the over-excitation of the photosynthetic apparatus and the probability of photo-inhibition to occur at high irradiance periods in the day, particularly at noon, but also reduce the photosynthetic light amount needed for carbon assimilation. However, coffee is able to maintain net photosynthesis at levels similar to full sun at up to 55% light reduction, because photosynthetic light saturation are reached at irradiances quite below FS values in leaves acclimated to high irradiance exposure (Ramalho et al., 2000; Franck et al., 2007). Additionally, a recent study corroborates the idea of photosynthetic maintenance in shaded-coffee plants (Charbonnier et al., 2017). Despite a 60% reduction in irradiance below the canopy of the shade trees, coffee plants grown under shade increased their light-use efficiency by 50% and the overall aboveground net primary productivity (leaves, fruit, wood, etc.) was not statistically different to that of the FS-grown plants. This demonstrates how coffee plants can potentially compensate for the reduction in solar irradiation in a shaded environment by increasing their photosynthetic efficiency. Martins et al. (2013) supported this hypothesis by demonstrating a comparable level of net photosynthesis in coffee plants grown under 90% shade cover compared to FS, when calculated on a mass basis. This study also determined difference in the kinetics of photosynthesis, showing that the shade grown leaves exhibited faster photosynthetic induction compared with their sun counterparts, likely explained as an adaptive response to use the light energy from sun-flecks. Such increased photosynthetic performance observed in shade-grown coffee may allow the plant to maximize the potential for carbon assimilation in a low light environment, and is likely associated to the forest-understory evolutionary origin of *C. arabica*.

Nevertheless, there are cases in which the well-illuminated leaves from the upper part of the coffee canopy showed greater net carbon assimilation rate (A), associated with higher electron transport rate, as compared to self-shaded, lower-canopy coffee leaves in the same plant (Araujo et al., 2008). This study also concluded that there was no major difference in stomatal and mesophyll conductance between sun and shaded coffee leaves, which were similar regardless of leaf position. However, morphological (e.g., variations in specific leaf area and leaf inclination) or anatomical plasticity is likely of greater value in terms of acclimation to low-light environments. When considering leaf age, Campa et al. (2017) found that only mature coffee leaves were capable of acclimating to high-light conditions for *C. arabica* cv. Naryelis (grown under controlled conditions). The growing and juvenile leaves (under development) were found to have inefficient photo-protection mechanisms, scarce antioxidant protection, and a poor ability to export sucrose under increasing light conditions. These observations suggest that despite the fact that the *C. arabica* Naryelis cv. was selected for FS conditions, immature leaves of this cultivar are somehow sensitive to high light levels. These findings are in line with other reports showing a high acclimation plasticity of newly-matured coffee leaves after transition from deep shade to FS exposure, associated with the reinforcement of photosynthetic components, anti-oxidative and photo-protective mechanisms, as well with changes in the lipid profile of chloroplast membranes (Ramalho et al., 1997, 1998, 2000). Interestingly, these high irradiance stress responses, which potentially exacerbate oxidative stress conditions (as a secondary stress), constitute a common response in coffee leaves to other stresses such as heat (Rodrigues et al., 2016), cold (Ramalho et al., 2014), and drought (Dubberstein et al., 2020), thus confirming plasticity of some elite coffee genotypes to environmental constraints that should be explored in terms of breeding purposes.

TABLE 2 | The effects of shade on beans size, biochemical and organoleptic attributes associated with coffee quality of *C. arabica* cultivars and *C. canephora* species.

	Bean size	Caffeine	Chlorogenic acids	Sucrose	Trigonelline	Lipids	Acidity	Aroma	Body	References
Bourbon	▲	▲	▲	▲	▼ ▼					Guyot et al., 1996
Catimor	▲		▲	▲	▲		▲	▼		Muschler, 2001, 2004; Somporn et al., 2012
Catuaí	▲	▲	▲	▲	▼				▲	Guyot et al., 1996
Caturra	▲			▼			▼	= ▼	▼	Muschler, 2001, 2004; Bosselmann et al., 2009
Costa Rica 95	▲	▲	▼	▲	▼	▲	▲			Vaast et al., 2006; Worku et al., 2018
IAPAR 59	▲	▲		▼ ▲	▼	▼				Geromel et al., 2008; Delaroza et al., 2017
K7	▲	▲	▲ ▲	▼	▲			▲ ▼		Alves et al., 2018; Cheng et al., 2020
C. canephora	▲ =	▲	▼	▼		▼		▲		Vaast and Raghuramulu, 2012; Odeny et al., 2014; Alves et al., 2018; Ehrenbergerová et al., 2021

▲, *increased;* ▼, *decreased;* =, *equivalent to full sun.*

Full-sun cultivation of coffee promotes a higher number of nodes and fruits per branch, leading to a greater competition between fruits, resulting in smaller beans, and biennial production due to heavy flowering followed by reduced flowering in the subsequent year (Cannell, 1974, 1976, 1985; DaMatta, 2004), although these problems can be mostly overcome with adequate fertilization and irrigation management under full sun cropping. In contrast, shaded-coffee production is synonymous with fewer nodes per branch, lower fruit loads and hence less competition between fruits, leading to larger beans, less alternate bearing pattern and less branch dieback (DaMatta, 2004). All these factors influence the annual yields and profitability of the coffee farm and are thus critical in the decision-making process regarding light/shade management. In particular, the flowering intensity has a direct influence on fruit loading in coffee plants (Franck et al., 2007). The Caturra cultivar of *C. arabica* was reported to produce a high variation in flower intensity across a spectrum of light, resulting in *ca.* 4,620, 3,052, 1,500, and 605 flowers per plant, under FS condition, 25, 50, and 75% light reduction, respectively. While shade has been reported to have a beneficial effect on bean size and density (Muschler, 2001, 2004; Morais et al., 2006; Vaast et al., 2006; Geromel et al., 2008; Bote and Struik, 2011; Somporn et al., 2012), the reduced flowering intensity under lowered irradiation in many cases leads to yield reduction (Vaast et al., 2006; Jaramillo-Botero et al., 2010). Therefore, coffee growers using shade-systems must balance the fine-line of negative effects (e.g., associated with flowering intensity) with the positive effects (e.g., lower need of water or fertilization inputs), to obtain a desired fruit load and bean quality. This is no easy feat and likely dependent on individual cultivar sensitivity to a low-light environment with respect to flowering, as well as to other environmental factors (i.e., whether the farm is operating under optimal/sub-optimal conditions of water availability and temperature, etc.) and/or the management the shade level through timely pruning of shade trees.

SHADE EFFECTS ON COFFEE QUALITY ATTRIBUTES

There is little literature devoted to shade effects on cup quality or on the biochemical composition of coffee beans (Leroy et al., 2006). Here, we define coffee quality in the context of the bean size, biochemical composition, as well as organoleptic attributes mainly for *C. arabica* and for *C. canephora* (when available), with the main interactions between shade and coffee quality attributes shown in **Table 2**. As the farmers' choice of cultivar is a critical factor influencing quality, we also assess shade effects at the cultivar level (where feasible).

Shade Effects on Coffee Bean Size

Bean size is an important parameter defining coffee quality, with large beans being frequently associated with better cup quality and high prices in international markets (Vaast et al., 2006; Sanz-Uribe et al., 2017). For Arabica, high percentages of large coffee beans were reported under both natural and artificial shade (**Table 2**). The effects of shade on coffee bean size can be explained by the fewer branches produced under shade, with smaller number of nodes per branch, and fewer numbers of flowers per node. These shade impacts contribute to a reduced fruit load under shade (Cannell, 1976; Morais et al., 2006; Vaast et al., 2006; Jaramillo-Botero et al., 2010). Moreover, shade also lowers the tree stress (explaining lower biannual bearing pattern) (Fahl et al., 1994), and hence favors slow fruit ripening, better filling of beans which increases bean size, and ultimately cup quality (Morais et al., 2006; Vaast et al., 2006; Bote and Struik, 2011; da Silva Neto et al., 2018).

Compared to shade studies on *C. arabica* coffee, the impacts of shade on *C. canephora* are much scarcer. Vaast and Raghuramulu (2012) showed that the effects of shade on Robusta bean size were largely dependent on the shade trees selected and rainfall conditions. Under low rainfall conditions, Robusta

intercropped with *Artocarpus heterophyllus*, *Dalbergia latifolia,* and *Lagerstroemia microcarpa* had a higher percentage of larger than normal bean size (coffee grading AA equivalent to >7 mm diameter) than those intercropped with *Grevillea robusta*. However, Robusta intercropped with *A. heterophyllus* or *G. robusta* provided lower AA bean percentage under high rainfall. Another study also reported that the AA bean percentage was strongly and positively influenced by the density of non-*Grevillea* shade trees but not by that of *G. robusta* (Boreux et al., 2016). In a more recent study performed across three Robusta plantations in Cambodia, Ehrenbergerová et al. (2021) showed that coffee bean size, as well as fruit ripening and yield, were not affected by shade trees.

Shade Effects on Coffee Bean Biochemical Composition

Although shade is widely documented to delay coffee bean maturity, knowledge of shade effects relating to coffee biochemical compounds is somewhat limited (Cheng et al., 2016). In green beans, coffee quality can be ascertained by quantifying some chemical compounds, namely sugars (e.g., sucrose), lipids, chlorogenic acids (CGA), and caffeine (Leroy et al., 2006). The light effects on sucrose are uncertain, since it was reported to increase both under FS (Vaast et al., 2006; Geromel et al., 2008; Delaroza et al., 2017; Worku et al., 2018), and shaded conditions (Guyot et al., 1996; Muschler, 2001, 2004; Somporn et al., 2012) (**Table 2**). A general negative relationship between fat and sucrose contents has been reported in both Arabica and *C. canephora* coffee species (Montagnon et al., 1998; Bertrand, 2002; Vaast et al., 2006). As sucrose is a precursor of polysaccharides and fat compounds (Bradbury and Halliday, 1990), Vaast et al. (2006) hypothesized that the high sucrose (as well CGAs and trigonelline) and low lipid contents (often observed under FS condition) pointed toward incomplete bean maturation, although a tendency to lower lipid content can result also from higher temperatures in mature beans (Ramalho et al., 2018).

The variation of bean biochemical contents observed under shaded vs. FS coffee beans is speculated to arise from differential enzymatic activities. In pericarp and perisperm tissues separated from *C. arabica* matured cherries, higher activities of sucrose synthase (EC 2.4.1.13) and sucrose-phosphate synthase (SPS: EC 2.4.1.14) were detected under shade as compared to full-sun (Geromel et al., 2006). These two enzymes also had high activity peaks in developing beans (endosperm) which could explain the sucrose reduction and the increase in reducing sugars observed under shade condition. By performing DPPH (1,1-Diphenyl-2-picrylhydrazyl) radical-scavenging activity assays, Somporn et al. (2011) reported that bean extracts of *C. arabica* cv. Catimor grown under shade had higher antioxidant activities than those from plants grown under FS.

In a more recent study on Robusta, Alves et al. (2018) analyzed the polyphenol oxidase (PPO) activity, an enzyme commonly considered as associated with good cup quality (Mazzafera, 1999). The results of sensorial analyses performed by these authors corresponded to PPO activities in beans with shade-grown coffee plants exhibiting lower PPO than those measured in beans harvested under FS condition.

The bean biochemical composition (and their associated cup quality) variation observed in shaded vs. FS cultivated coffee plants has been attributed to greater expression of genes involved in important metabolic pathways (de Castro and Marraccini, 2006; Cheng et al., 2020). For example, the elevated sucrose level observed in lower canopy (LC) beans compared to the upper canopy (UC) beans is likely the result of increased expression of a battery of genes (such as *SPS1*, *SPS2.2*, *SUS2.1*, *CIN*, *VIN1*, and *VINI.2*), previously reported to play a key role in coffee bean sugar metabolism (Geromel et al., 2006; Privat et al., 2008; Joët et al., 2009; Marraccini, 2020). Another example of this is the elevated expression of *RD22* (a gene involved in plant responses to stress as reported by Yamaguchi-Shinozaki and Shinozaki, 1993) in beans from LC compared to UC, suggesting that the continued bean growth until the red stage in LC was facilitated by a stronger dehydration resistance and less chlorophyll degradation. Today, we are closer to characterize candidate genes (CGs) associated with the slow bean maturity process (Cheng et al., 2020). By selecting genotypes or environments (such as AFS) which are able to increase the expression of such CGs, higher quality coffee may be obtained, mitigating the adverse forecasted effects of climate change.

Shade Effects on Organoleptic Attributes

The previous section showed that shade influenced the biochemical composition of coffee beans. Among these compounds, it is well-known that sucrose, caffeine and trigonelline are essential flavor precursors able to form flavor components after roasting (Grosch, 2001; Homma, 2001). For example, caffeine is associated with the strength, body, and bitterness of coffee beverage and trigonelline is strongly correlated with high coffee quality (Farah et al., 2006; Janzen, 2010). The genetic origin (species and cultivar) of coffee plants can greatly influence the final cup quality (Bertrand et al., 2003, 2006; Leroy et al., 2006; Montagnon et al., 2012). The environmental conditions of coffee cultivation are also key drivers of cup quality (Bertrand et al., 2012). Positive quality attributes such as acidity, fruity character and flavor quality were found to be correlated and typical of coffees produced at cool climates and higher elevations (Bertrand et al., 2012). Therefore it is a great concern that increasing temperatures (associated with predicted climate change) will likely lead to negative impact on mid and lowland coffee quality. Worku et al. (2018) reported that the acidity of coffee grown under shade increased by 0.22 points for each 100 m increase in altitude, while no altitude effect on cup acidity was reported for coffee grown without shade. Acidity, trigonelline and total CQA content were reported to significantly increase in green beans under higher temperatures (Ramalho et al., 2018). Altogether, these findings suggest that the drift of these compounds under changing temperatures might be predominantly genotype-related. In a more recent study also performed in Ethiopia, *C. arabica* grown under diverse forests covers and densities (Tassew et al., 2021), also reported that cup quality organoleptic attributes

(acidity, body, raw total, flavor, and cup total values) as well as grade were significantly and positively affected by shade and increasing elevation.

Several studies have highlighted the positive effects of shade on coffee cup quality (**Table 2**). On the contrary, a negative effect of natural shade on cup quality (fragrance, acidity, body, and sweetness) was reported for the *C. arabica* cv. Caturra grown at high altitudes (between 1,439 and 1,629 m.a.s.l.) in Southern Colombia (Bosselmann et al., 2009). This negative effect was likely due to the relatively high altitude of the study site, suggesting that the shaded environment promoted a lower than optimal air temperature for coffee production. Negative effects of shade on cup quality were also reported by Tolessa et al. (2017) when investigating the effects of both altitude and shade on the quality of Ethiopian specialty coffee. Their results suggested that the changes of quality scores driven by altitude, shade, and harvest period were small, but nonetheless led to re-classification of speciality coffee (Q1: SCAA note $\geq$85) to a lower classification (Q2: SCAA note 80–84.75) resulting in a lower international market price for farmers.

At the field level, the spatial distribution of both coffee and shade trees also influences the coffee bean biochemical composition (Delaroza et al., 2017) and consequently its cup quality. When studying the spatial distribution of different shade trees (*Anadenanthera falcata, Albizia polycephala,* and *Cassia grandis*), da Silva Neto et al. (2018) observed that the distance between coffee plants and shade trees affected the cup quality of beans from *C. arabica* cv. Obatã Vermelho. The best coffee cup quality was observed when coffee was harvested ∼1 m from the trunk of the shade trees.

Shade effects on quality act through complex interactions with environmental (e.g., altitude/temperature), shade trees and processing (e.g., post-harvest treatments) factors, especially in sub-optimal zones (Walyaro, 1983; Joët et al., 2010; Geneti, 2019; Hameed et al., 2020). While positive effects of shade on coffee quality are now well-recognized for Arabica, few articles were dedicated to show the impact of shade on *C. canephora* cup quality. The shade effects on *C. canephora* coffee have recently been examined and shown to positively correlate with growth, yield, physiological, photosynthetic, ecological and microclimatic variables (Venancio et al., 2019; Piato et al., 2020). However, shade cover beyond 30% was associated with reduced beverage quality in the same review. When studying the effects of shade composition on Indian Robusta coffee quality, Vaast et al. (2011) reported that increasing the percentage of *Grevillea robusta* exotic species resulted in a decrease in cup quality as well as aroma and body. In a recent study, sensorial analyses performed by Alves et al. (2018) revealed that roasted-ripe beans of Brazilian *C. canephora* (known as "Conilon") grown under rubber (*Hevea brasiliensis*) shade trees produced a lower cup quality than those grown under FS. The authors noticed the superior quality of sun-grown Conilon beans was also accompanied with higher lipid, total sugar contents, PPO activity and lesser membrane damages than under shade. Altogether, these observations led the authors to propose that shade-grown Conilon beans have undergone micro-organismal activity and/or undesired (or excessive) fermentation during cultivation, which resulted in lower coffee quality. Given the potential negative impact on cup quality, the use of densely shaded cultivation practices is not recommended for *C. canephora*. However, much work remains in order to characterize the cultivar and/or environmental dependency of bean quality changes, both in Arabica and, especially, in Robusta clones.

BREEDING OF NEW ARABICA COFFEE F1 HYBRIDS FOR FUTURE AGROFORESTRY SYSTEMS

Coffee cup quality clearly depends on coffee genotypes and on the genotype-environment interactions (Moschetto et al., 1996; Bertrand et al., 2006; Montagnon et al., 2012; Cheng et al., 2016). Historically, coffee breeding efforts have been mostly geared toward higher yield, as well as to pest and disease resistance (Lashermes et al., 2009; Bertrand et al., 2011), inadvertently at the expense of quality. Paradoxically, the demand for specialty, high quality, and sustainably sourced coffee has dramatically increased in the last decade (van der Vossen et al., 2015). These consumer trends call for coffee breeding efforts geared toward improving coffee quality and production under sustainable AFS conditions.

Over the last three decades, a new generation of Arabica coffee hybrids has been developed for a sustainable production of coffee (Bertrand et al., 2019). Unlike previous breeding programs, focused on high yielding varieties with CLR resistance (e.g., Lashermes et al., 2009; Bertrand et al., 2011), recent breeding strategies have extended the selection of Arabica hybrids to include high cup quality and adaptation to shaded systems (van der Vossen et al., 2015; Georget et al., 2019). These modern Arabica breeding programs widen the very narrow genetic base of cultivated American coffee cultivars by crossing them with wild Ethiopian germplasm (Engelmann et al., 2007; van der Vossen et al., 2015). Such crosses of cultivated lines (such as the Sarchimors) with wild pools often results in hybrid vigor where the offspring (F1-hybrids) exhibit higher vegetative growth and yield than its two parents (Fu et al., 2014). A quick recovery process of genes (in a single cross), which has also shown to be involved in adaptation to shade has recently been explored and proved to be successful when selecting ombrophilous Ethiopian individuals as the male parent to the F1 hybrids (van der Vossen et al., 2015; Georget et al., 2019).

Arabica F1 coffee hybrids have shown a better agronomic performance, yield and cup quality, and higher bean size compared to their cultivated Sarchimor parental lines (Bertrand et al., 2019; Marie et al., 2020). In Costa Rica, F1 hybrids produced between 11 and 26% more coffee yield compared to their parents and had an 8–10% increase in the 100-bean weight (Bertrand et al., 2005). Across a Central American network of 15 field trials combining FS and AFS systems, these hybrids demonstrated average yields that were 37% higher than the best commercial varieties, such as Caturra and Catimor, in both systems (Bertrand et al., 2006, 2011). Moreover, these high yields and quality parameters were more stable over varying environments, climates and production cycles, thus confirming their homeostasis and

potential for acclimation to future climate conditions. When comparing performance under AFS conditions, the hybrids produced 50% more green beans than commercial pure line varieties (Bertrand et al., 2011). In culture chambers, F1 hybrids have also demonstrated a higher resistance to CLR especially when under low light conditions compared to commercial, pure line varieties, even when coupled with a low nitrogen supply as often observed in agroforestry conditions (Toniutti et al., 2017). It was recently proposed, that an altered circadian clock coupled with a higher photosynthetic efficiency could explain the superior agronomic performance of hybrids and their higher homeostasis (Toniutti et al., 2019; Breitler et al., 2020).

Molecular indicators and/or predictors, which can characterize shade-adapted coffee genotypes, are currently being identified. For example, it has been demonstrated that a high content of specific secondary metabolites such as chlorogenic acid 5-CQA and xanthone mangiferin indicates an adaptability to shade in coffee plants (Duangsodsri et al., 2020). Moreover, 5-CQA and mangiferin leaf contents, in full sun and shade, allowed for differentiating the genetic groups of Ethiopian wild accessions (higher contents) vs. cultivated American pure lines. This may be linked to the origin of the Ethiopian wild accessions, i.e., understory bushes in mesophilous forests (Sylvain, 1955) and that of American pure lines derived from the *C. arabica* "Yemen-Harare" group domesticated for cultivation in full sunlight (Scalabrin et al., 2020). The Arabica F1 hybrids had very similar concentrations of 5-CQA and mangiferin in the leaves of shaded and unshaded plants to those measured in their mother American pure lines, suggesting a significant maternal effect in adaptation to light growing conditions. This should be considered in future breeding programs. Such biochemical indicators will strengthen research around the potential for shade adaptability of coffee, allowing the development of physiological models of functioning under shade and can be exploited as potential markers to control the selection of more high-yielding varieties suitable for agroforestry. For this breeding purpose, all the genetic resources and the Arabica hybrids under selection must be placed under shade and this, from the juvenile stages in the nursery.

Using the new Arabica coffee F1 hybrids as a case study, the BREEDCAFS project [Breeding Coffee for Agroforestry Systems (2017–2021), https://www.breedcafs.eu/] has tested coffee varieties under AFS conditions. This project also characterized and identified the main response mechanisms of the coffee plant under several stressful conditions (e.g., heat, drought) and the potential mitigation role of eCO_2 against stress impacts. Some of these new hybrids have been found to be better adapted and suited for climate change scenarios, while maintaining cup-quality and a robust defense system to biotic and abiotic stresses. The large-scale production and distribution of F1-hybrids is ongoing to provide farmers with high-quality and affordable seedlings. New horticultural solutions like mini-cuttings (Georget et al., 2017; Etienne et al., 2018) and seed gardens using mother plants with male sterility (Georget et al., 2019) are making the F1 hybrids more accessible to coffee farmer communities who often live in remote mountainous areas.

Unlike Arabica coffee, breeding programs geared toward *C. canephora* coffee currently do not select for shaded cultivation systems (Carvalho et al., 2019; Alkimim et al., 2021). Moreover, selection criteria for AFS were not clearly established until Bertrand et al. (2021) proposed a list of target traits including productivity, size of the beans, sensory score, and coffee tree volume allowing a high number of trees per hectare. Applying these criteria, they found hybrids capable of producing up to 22% more than the best pure line under full sun. Nevertheless, selecting for AFS requires years of phenotyping data and are quite time and money consuming. By combining extensive phenotyping with eco-physiological, metabolomic and transcriptomic studies, analytical, and predictive tools for coffee genomic selection have been recently developed (Mbebi et al., 2021). This has led to marker-aided rapid selection and a novel approach for breeding of perennial crops.

If climate change leads to extreme temperature increases, the extensive gene pool of the more than 120 wild species (Armarego-Marriott, 2021) may be researched and exploited in breeding programs aiming for more climate resilient coffee plants. Currently, only the two species *C. arabica* and *C. canephora* are cultivated. The idea was given recently by Davis et al. (2021) who reported that *C. stenophylla*, a wild species from Upper West Africa reveals a superior flavor and a sensory profile analogous to high-quality Arabica coffee. These authors demonstrated that this species grows at a mean annual temperature 6.2–6.8°C higher than Arabica coffee. Another example is *C. racemosa* Lour. (formerly known as *C. ibo*) which received a golden medal in a Lisbon fair in 1906 due to unique characteristics of taste and aroma, considered the best coffee of all Portuguese colonies (Vasconcellos, 1906), being present in lowlands (below 200 m) and areas of low water availability in Mozambique (Hallé and Faria, 1973). The valorization of these wild species could result in the selection of a large number of interspecific hybrids adapted to much hotter and drier climates as well as to shade.

COFFEE PRODUCTION UNDER CLIMATE CHANGE

Some climate model scenarios estimate a 50% decrease in the global area suitable for coffee production by 2050, which can reach up to 85% reduction in Brazil alone (Davis et al., 2012; Baca et al., 2014; Bunn et al., 2015a,b). This loss of adequate areas was estimated to be accompanied by aggravated incidence of pests and diseases (Magrach and Ghazoul, 2015), severe yield drops (van der Vossen et al., 2015), and even the extinction of a large number of wild coffee species (Davis et al., 2019). Still, negative climate impacts in coffee producing regions can be attenuated through climate adaptation measures. These include research, extension, and credit subsidies for improved coffee varieties, adequate irrigation, and the implementation of AFS management and ultimately considering the possibility of crop substitution (Koh et al., 2020). However, some coffee genotypes have demonstrated an intrinsic resilience to environmental

constraints (Dubberstein et al., 2020; Semedo et al., 2021) than traditionally anticipated, but in line with the somewhat harsh environmental conditions endured by the plants under full sun conditions (DaMatta and Ramalho, 2006). Furthermore, a growing body of recent evidence shows that the increasing concentration of atmospheric CO_2 (usually associated to global warming), can actually improve the coffee plant photosynthetic performance (Ramalho et al., 2013; Ghini et al., 2015), promote carbon investment in reproductive structures (Rakocevic et al., 2020), and boost yield under adequate rain fed (or irrigated) water supply (DaMatta et al., 2019; Pham et al., 2019). More importantly, eCO_2 was shown to strengthen the resilience to supra-optimal temperatures on both *C. arabica* and *C. canephora* plants up to ∼37°C or even 42°C (Martins et al., 2014, 2016; Rodrigues et al., 2016; Marques et al., 2021). This was associated with a reinforcement of photochemical efficiency, biochemical functioning, and protective mechanisms (Martins et al., 2016; Rodrigues et al., 2016; Scotti-Campos et al., 2019; Avila et al., 2020a,b). Although eCO_2 can alter the content of some compounds in the coffee bean (Marcheafave et al., 2020; Rakocevic et al., 2021), it may also have a positive role in the preservation of bean quality under heat stress (Ramalho et al., 2018). Recent reports show the combined effects of high light and eCO_2 in improving coffee growth and photosynthetic performance (Marçal et al., 2021). However, allometric and biomass partitioning of the coffee plants is affected by combined high light and eCO_2 treatments, most commonly resulting in a higher root biomass-to-total leaf area and lower leaf area ratio (Avila et al., 2020a; Marçal et al., 2021). Other studies have attempted to mimic high atmospheric demand (such as elevated vapor pressure deficit) under non-limiting soil-water supply (Machado Filho et al., 2021). Genotypic variation was observed in root and stem hydraulic conductances and conductivity, as well as whole plant conductivity as a coping mechanism for elevated vapor pressure deficit in coffee. Studies such as these allow for physiological predictions of how elevated temperatures can affect coffee in irrigated farming systems.

Additionally, eCO_2 was recently reported to have also marked implications on how coffee plants respond to soil water deficit, greatly attenuating drought impacts (even under severe water deficit conditions) regarding, among others, the photosynthetic performance, hydraulic conductance, and growth, as well as gene expression and metabolite profile (Avila et al., 2020a,b; Fernandes et al., 2021; Rodrigues et al., 2021; Semedo et al., 2021). When eCO_2 is combined with a single drought episode, high genotypic heterogeneity has been observed in the primary metabolite responses of both *C. arabica* and *C. canephora* cultivars (Rodrigues et al., 2021). These findings stress the phenotypic plasticity of the *Coffea* genome and how it can be harnessed through targeted climate adaptation breeding programs. Moreover, improved modeling approaches (namely integrating the eCO_2 "fertilization" effect) can constitute a powerful tool to assist coffee cultivation under climate change (Rahn et al., 2018). By designing shade management strategies (i.e., selecting the right tree species according to the local context and regulating shade level along an altitudinal or rainfall range) coffee systems can be adapted to climate change at landscape scale. Despite recent projections by Moat et al. (2017), showing an overall negative impact of climate change on the Ethiopian coffee sector, the recent findings on eCO_2 and the use of elite coffee genotypes reviewed here support a less grim perspective than earlier forecasted by modeling approaches eluded to (largely based on temperature drifts) (DaMatta et al., 2019). In this context, continued breeding for improved shade tolerance and climate adaptability will likely further improve land-use prospects for coffee cultivation.

CONCLUDING REMARKS

We started out this work in order to shed light on whether shaded-coffee is a feasible, nature-based solution for climate change in coffee production. In order to answer this enquiry, we took a comprehensive look at the coffee farm, starting with the farmer's use of shade and global perceptions of the services of shade trees across the coffee belt. We then focused on the aboveground interactions of the shaded coffee microenvironment (including effects on the air temperature and water-use) as well as pest and disease incidence. The carbon assimilation and phenology of coffee plants was examined under shaded environments as these aspects directly relate to coffee yields. The development of coffee quality attributes under shade is highly contentious and thus also present in this evaluation of the coffee-AFS. Breeding trends and directions were considered in relation to Arabica F1 hybrids and Robusta clones under AFS. Finally, we evaluated the newest data pertaining to coffee production under climate change in order to determine the most relevant physiological limitations of the coffee plant under future climates.

Many smallholder coffee farmers demonstrate a sound expertise in shade management for coffee production throughout the seasons to optimize and counteract farm humidity, light interception and fungal pathogen attacks. By utilizing shade, coffee farmers may also reap the associated benefits including increased biodiversity, biological control of pests and diseases, climate-buffering services, as well as diversified incomes resulting from shade-tree products. While there has been a general decrease in coffee AFS over the last couple of decades, the growth in demand for specialty, certified coffee, combined with consumers' increased concern for sustainability and farmers' need to adapt to climate change, could reverse the trend, and favor the uptake of more coffee AFS in the future.

Shaded-coffee systems are shown to alter both the above- and belowground microenvironment, and impact the physiology, phenology (and therefore yield) as well as quality attributes of coffee. Recent work examining the shade effects (associated with AFS) on the coffee micro-environment tended to corroborate past studies which showed reductions in air temperatures, wind speeds, radiation levels, weeds, coffee water transpiration losses, and protection from frost events.

Regarding the physiology and phenology of coffee plants under shade, the photosynthetic performance of coffee plants can potentially compensate for the reduction in irradiation under shaded-environments; however, a lowered flowering intensity

can lead to yield reductions if the shade level is too high. These physiological aspects could be taken into account also in future breeding efforts, in order to reduce negative shade effects in new genotypes.

In terms of the shade effects on coffee diseases and pests, many of the recent findings appear to be locally specific to their environments and/or the shade tree species used, hence a large variation was observed by a number of studies in this context. Pest or pathogen management advice should be as local as possible and linked to weather forecasting (especially precipitation and humidity) in order to be relevant to the coffee farming region.

Several cup quality and/or biochemical attributes of coffee were also shown to evolve positively under shade on *C. arabica* cultivars such as Catuaí, Catimors (especially CR95), and Sarchimors (especially IAPAR59), although for some cultivars no sensible advantages arise from shade implementation. Moreover, recent Arabica breeding programs have developed F1-hybrids able to maintain high yield and cup quality under shaded conditions. In the case of *C. canephora* coffee, preliminary research has pointed toward a decrease in quality aspects under shaded environments. Moreover, current breeding trends for *C. canephora* do not prioritize selection for shade, but could be oriented toward breeding for high-temperature-resistant clones.

Studies examining the impact of eCO_2 on the coffee plant are quite new (within the last decade), but have already shown that eCO_2 can improve coffee plant photosynthetic performance, promote carbon investment in reproductive structures, boost yield, and, especially important can increase plant vigor and its resilience to heat and drought constraints. However, the coffee plant is still highly vulnerable to the other detrimental impacts associated with climate change, thus new management practices must be considered for future production.

Overall, coffee AFS should match farmers' needs along with risk assessments of climatic hazards specific to the local environment. When AFS are site-specific in this way they can act as an adaptation strategy against climate change. The buffering effects, which AFS have on the coffee microclimate, serve as a mitigation against unfavorable environments or climate change events. Given this, coffee farmers may in fact be better off managing an AFS compared to intensive FS systems. Finally, the array of ecosystem services together with alternate revenue streams and increased cup qualities (for Arabica), provided by shade trees, may help compensate for potential yield losses under coffee AFS.

In order to correct the geography bias evident in the literature, an expansion of AFS-coffee studies is encouraged to take place in South East Asia (e.g., Vietnam and India) as well as in Africa (e.g., Kenya, Tanzania, and Uganda). This would allow for the examination of coffee-AFS across different cultivation environments and eventually lead to the implementation of management practices, which are both culturally and locally relevant. Future research should continue the development of coffee varieties adapted to agroforestry systems, in particular those which can maintain a high level of yield under shade. This can help to improve the overall profitability of coffee plantations in AFS. Research must continue to refine extension tools including the selection of locally-adapted shade tree species and mobilize their widespread use by coffee farmers. Other management practices can also be optimized along the coffee production cycle such especially concerning shade tree management (i.e., timely pruning). Finally, multi-faceted approaches which consider the market, social, and policy issues must also come into play in order to provide necessary recommendations to enable the adoption of AFS in coffee cultivation. An example of this is an incentivized scheme for the renovation of existing coffee farms using AFS together with new Arabica hybrid varieties bred for the AFS environment.

AUTHOR CONTRIBUTIONS

Conceptualization was conducted by AK, PM, and AR. Literature review was conducted by AK and supplemented by all authors. Writing (original draft preparation) was conducted by AK, AB, CC, CR, HE, JR, MB, NT-G, PM, PV, and TS. All authors contributed to the writing (review and editing) and approved the final version of the manuscript.

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Comprehensive Composition of Flavor Precursors in Kopi Luwak and Jacu Exotic Green Bioprocessed Coffees

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Exotic coffees may be defined as extravagant and unique coffees, primarily due to their production mode, including unusual bioprocessing or fermentation conditions associated with superior sensorial characteristics. The aim of the present study was to investigate the influence of bioprocessing and of growing conditions on flavor precursors of Jacu and Kopi Luwak exotic green coffees, respectively. Moreover, this is the first study to perform a detailed chemical analysis of these exotic coffees. Thirteen green *Coffea arabica* bean samples were obtained, five from Espírito Santo state, Brazil, and eight Kopi Luwak from different regions of Indonesia. Samples were analyzed regarding their proximate composition, chlorogenic acids (CGA), sucrose, alkaloids, triacylglycerols (TAG), diacylglycerols, free fatty acids, sterols, diterpenes and tocopherols. Scanning electron micrography confirmed bioprocessing of Jacu and Kopi Luwak coffee samples. Bioprocessing by the Jacu bird caused reductions of 69 and 28% in caffeine and CGA contents, respectively. The TAG profile of Jacu coffee was modified. TAG containing two saturated fatty acids were preferably hydrolyzed in detriment to those containing two unsaturated fatty acids. Other coffee components were not affected by the bird's digestion of the beans. Kopi Luwak coffee samples had a chemical composition in accordance with reported ranges for non-bioprocessed green *C. arabica* samples, except for caffeine (0.48 g/100 g) and CGA (5.09 g/100 g), which were found in low amounts. Crop year rather than location or post-harvest processing discriminated Kopi Luwak coffee samples, suggesting that weather conditions would be the most crucial aspect for their chemical composition, especially in terms of total lipids, ashes, total CGA, sucrose and proteins.

Keywords: caffeine, chlorogenic acids, diterpenes, lipids, proximate composition, sterols, trigonelline

INTRODUCTION

Exotic coffees may be defined as extravagant and unique coffees, primarily due to their production mode, including unusual bioprocessing or fermentation conditions which leads to peculiar and superior sensorial characteristics (Lee et al., 2015). Kopi Luwak is the most commercialized and well-known exotic coffee. For its production, beans cultivated in Indonesia are ingested by the Asian palm civet (*Paradoxurus hermaphroditus*), being digested in their gastrointestinal tract and excreted in their feces (Marcone, 2004; Lee et al., 2015; Ellis et al., 2016; Jumhawan et al., 2016; Arboleda, 2018; Hadipernata and Nugraha, 2018; Burns and Walker, 2019; Muzaifa et al., 2019; Tawali and Laga, 2019). Then, beans are cleaned and undergo the usual steps of either dry or wet post-harvest processing and roasting. Another bioprocessed exotic coffee is Jacu coffee, produced in Brazil and whose beans are ingested and excreted by the Jacu bird (*Penelope superciliares*), following dry post-harvest processing and roasting (Conti et al., 2013; Malacarne et al., 2017).

Exotic coffees have high selling prices (from USD 340 to USD 2,400 per kilogram), up to 100 times higher than the average, a direct consequence of its rarity and exclusiveness, but also to its complex production, which hypothetically adds a distinct and appreciated flavor to the product. The idea is that the animals select the ripest coffee cherries, which contain flavor precursors—caffeine, trigonelline, sugars, lipids, proteins, and chlorogenic acids (CGA)—that may be modified due to digestive conditions, including acid and neutral pH media (ranging from 2.7 to 6.4 for the Jacu bird and from 2.5 to 7.6 for the civet mammal), hydrolytic enzymes and microbiota, potentially impacting the volatile compounds produced during roasting (Lee et al., 2015; Ongo et al., 2015). According to Marcone (2004), bioprocessing associated with the digestion by the Asian palm civet attributes a unique flavor to Kopi Luwak coffee beans, described as earthy, musty, syrupy, smooth, and rich with both jungle and chocolate undertones.

Although some studies in the literature deal with the composition of exotic coffees, usually the approach is fragmented since limited classes of compounds are generally investigated. One of the first papers dealing with exotic coffees was published by Marcone (2004), which determined the proximate and microbiological composition of exotic civet coffees (Kopi Luwak, Nekemte Civet, and Abdela Civet), as well as of their controls (non-bioprocessed beans). Results suggested that during bioprocessing proteolytic enzymes penetrate the beans and caused proteolysis, impacting on coffee flavor. Conti et al. (2013) compared the contents of moisture, ash, protein, lipid, carbohydrate, total phenolic, 5-caffeoylquinic acid (the main CGA present in coffee), trigonelline, nicotinic acid and caffeine from roasted exotic (Kopi Luwak and Jacu), special (Gourmet and Premium) and traditional coffees. Cheong et al. (2013) analyzed the volatile composition of Asian coffees from different locations, including Kopi Luwak coffee, as well as studied the phenolic composition, sensory analysis and antioxidant activity. More recently, Ongo et al. (2015) applied the eletronic nose technique to geographically distinguish samples of Kopi Luwak coffee from different regions of the Philippines. It should be highlighted that there are only two studies dealing with the effect of Kopi Luwak coffee bioprocessing on the composition of flavor precursors (Marcone, 2004; Muzaifa and Hasni, 2016), and no investigation is available concerning the effect of Jacu coffee bioprocessing. The impact of growing conditions on the coffee flavor precursors has also been poorly studied, with only one study describing the effect of geographic origin on the non-volatile composition of Asian coffees, among them Kopi Luwak (Cheong et al., 2013).

Most studies, however, pay little attention to the lipid profile, which may significantly impact the flavor of exotic coffees (Muzaifa et al., 2019). This class has been related to coffee body, creaminess, and foam formation. Other flavor precursors such as caffeine, trigonelline and chlorogenic acids have also been related to coffee aroma (Cheng et al., 2016). Caffeine seems to provide strength, body and bitterness to coffee beverage, while trigonelline is associated to the overall aromatic perception and bitterness. Finally, chlorogenic acids attribute acidity, astringency and bitterness to coffee beverage.

In this context, in addition to determining proximate composition, alkaloids, sugars, and CGA, the profile of lipids was comprehensively analyzed, aiming to investigate the influence of bioprocessing and of growing conditions on these flavor precursors of Jacu and Kopi Luwak exotic green coffees, respectively.

MATERIALS AND METHODS

Standards and Chemicals

Zinc acetate and potassium hexacyanoferrate (II) were acquired from Merck (Darmstadt, Germany). Analytical standards of trigonelline (98.5%), caffeine (99.0%), sucrose (99.5%), and 5-caffeoylquinic acid (5-CQA) (95.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical standards of fatty acids were purchased from Supelco (Bellefonte, PA, USA; all 99% purity). Analytical standards of sterols (campesterol, 65%; β-sitosterol, 40%; stigmasterol, 95%) and triacylglycerols (1,2,3-triheptadecanoylglycerol and 1,2-distearoyl-3-palmitoyl-rac-glycerol, ∼99%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Coffee diterpenes were isolated and purified (purity > 97%) from Brazilian green Arabica coffee as described in previous work (Novaes et al., 2020). HPLC solvents were purchased from Tedia (Fairfield, OH, USA). HPLC grade water (Milli-Q system, Millipore, Bedford, MA, USA) was used throughout the experiments.

Coffee Samples

Five organic green *Coffea arabica* samples were obtained from Camocim Organic Farm, located at Domingos Martins, Espírito Santo state, Brazil (20°23'40.39"S, 41° 1'37.31"W) (**Figure 1B**). Two of these samples were bioprocessed by the Jacu bird (Jacu coffee), while the other three were not bioprocessed (non-Jacu coffee). Two samples were harvested in 2016: one Jacu coffee (dry-processed, dp) and one non-Jacu coffee (wet-processed, wp). Another three samples were harvested in 2017: one Jacu coffee (dp) and two non-Jacu coffees (dp and wp).

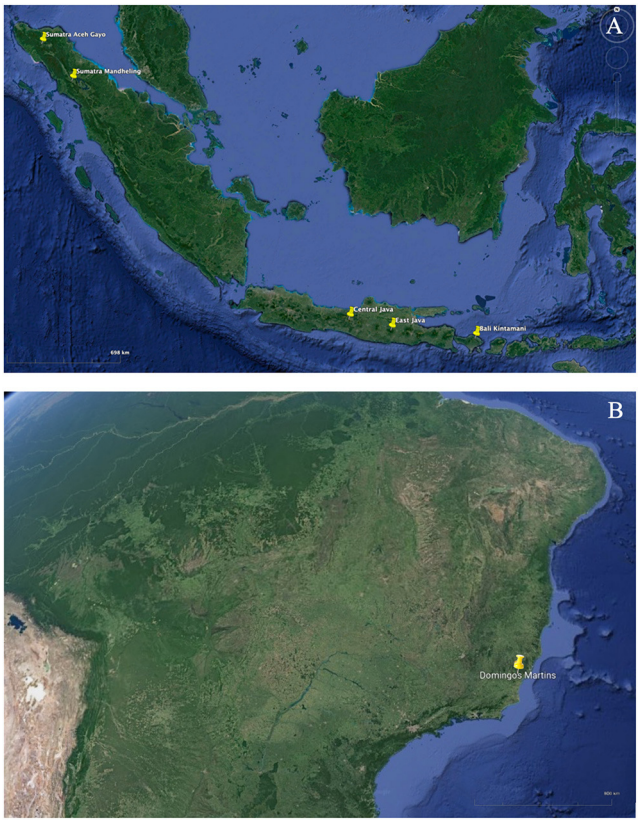

FIGURE 1 | Regions of Indonesia **(A)** and Brazil **(B)** from where Kopi Luwak and Jacu samples were obtained, respectively.

Eight non-organic Kopi Luwak green *C. arabica* samples of good quality (no coffee defects) from different regions of Indonesia (East Java, Central Java, Sumatra Aceh Gayo, Bali Kintamani, and Sumatra Mandheling) (**Figure 1A**) were purchased from Kora Coffee Import Company. Four samples were harvested in 2015 (East Java, Sumatra Aceh Gayo, Bali Kintamani and Sumatra Mandheling) and the other four in 2017 (Central Java, Sumatra Aceh Gayo, Bali Kintamani and Sumatra Mandheling). Sumatra Aceh Gayo and Sumatra Mandheling samples were dry-processed (dp), while Bali Kintamani, East Java, and Central Java samples were wet-processed (wp).

The harvest region, crop, post-harvest processing, and production mode of all green coffee samples investigated in the present study are described in **Table 1**. All samples were stored at −20°C until analyses.

Scanning Electron Microscopy

To confirm that Kopi Luwak and Jacu coffee samples were bioprocessed, beans were analyzed by Scanning Electron Microscopy (SEM) under conditions similar to that described by Marcone (2004). Beans were fixed at an aluminum apparatus with a double-sided carbon tape and coated in silver on an SCD005 Sputter Coater metallizer (BAL-TEC, Germany) under ultrapure argon for 250 s. Following coating, beans were viewed under a TM 3030 Plus scanning electron microscope (Hitachi, Japan) with a high voltage setting of 15 kV and magnification of 100 times. Prior to analyses, each sample was frozen in liquid nitrogen and ground to pass through a 0.46 mm sieve.

Proximate Composition

Moisture, protein, lipid, dietary fiber, and ash contents of ground green coffee beans were determined in triplicate, according to official methods (AOAC, 2000). Carbohydrate content was estimated by difference.

Chlorogenic Acids

CGA extraction was performed according to Trugo and Macrae (1984) in triplicate. An aliquot of 0.2 g of ground green coffee was added to 100 mL of boiling water and shaken at 300 rpm for 15 min in a water bath at 100°C. The mixture was centrifuged for 10 min at 1,500 *g*, and the supernatant was filtered through a filter paper (Whatman n° 1). The extract was clarified by adding Carrez's solutions, 0.3 M $K_2Fe(CN)_6$ and 1.0 M $Zn(OAc)_2$, and the final volume was made up with water to 100 mL. After 15 min, the colloidal suspension was filtered through a filter paper (Whatman n° 1) and kept at −20°C until LC-DAD analysis.

Chromatographic analysis was performed according to Perrone et al. (2008a). The LC system (Shimadzu, Kyoto, Japan) was comprised of a LC-10AD*vp* quaternary pump, a CTO-10AS*vp* column oven, an 8,125 manual injector (Rheodyne), with a 5 μL loop and a SPD M10A*vp* diode array detector (DAD) recording from 190 nm to 370 nm. The chromatographic separation was achieved by a C_{30} reverse-phase column (150 × 2.0 mm, 5 μm, Michrom BioResources, Auburn, WA, USA) maintained at 40°C. The mobile phase used was a gradient between 0.3% aqueous formic acid (eluent A) and methanol (eluent B) at a flow rate of 0.2 mL/min. Before injection, the column was equilibrated with 17% B for 10 min. Immediately after injection, this proportion was changed to 60% in 14 min until the end of the run in 35 min. CGA were quantified by external standardization (absorbance at 325 nm) using 5-CQA standard, corrected with each molar extinction coefficient. The method was previously validated by Farah et al. (2005). The calibration curve ranged from 0.1 to 20 ppm and its coefficient of determination (R^2) was 0.9993. The method is similar to that previously validated by Badmos et al. (2019).

Sucrose

Sucrose extraction was performed according to Perrone et al. (2008b) in triplicate. An aliquot of 0.2 g of ground green coffee was added to 60 mL of boiling water and shaken at 300 rpm for 15 min at room temperature. The mixture was filtered through a filter paper (Whatman no. 1). The extract was clarified by adding 2 mL of aqueous basic lead acetate (20%), and the final volume was made up with water to 100 mL. After 15 min, the colloidal suspension was filtered through a filter paper (Whatman n° 1) and kept at −20°C until HPLC-Evaporative Light Scattering detection (ELSD) analysis.

Chromatographic analysis was performed according to Kimball et al. (2004). The LC system (Shimadzu, Kyoto, Japan) was comprised of a LC-20AT quaternary pump, a 7725i manual

TABLE 1 | Harvest region, geographic coordinates, altitude, crop year, postharvest processing, and production mode of the green coffee samples investigated in the present study.

Sample	Harvest region	Geographic coordinates	Altitude (m)	Crop year	Postharvest processing	Production mode
Kopi Luwak	Sumatra Aceh Gayo, Indonesia	4°30'11.0"N, 96°44'35.5"E	~1,500	2015	Dry-processed	Non-organic
Kopi Luwak	Sumatra Aceh Gayo, Indonesia	4°30'11.0"N, 96°44'35.5"E	~1,500	2017	Dry-processed	Non-organic
Kopi Luwak	Sumatra Mandheling, Indonesia	2°44'37.3"N, 98°18'47.9"E	~1,100	2015	Dry-processed	Non-organic
Kopi Luwak	Sumatra Mandheling, Indonesia	2°44'37.3"N, 98°18'47.9"E	~1,100	2017	Dry-processed	Non-organic
Kopi Luwak	East Java, Indonesia	7°48'50.2"S, 111°45'36.0"E	~2,500	2015	Wet-processed	Non-organic
Kopi Luwak	Central Java, Indonesia	7°20'21.5"S, 110°01'31.2"E	~1,500	2017	Wet-processed	Non-organic
Kopi Luwak	Bali Kintamani, Indonesia	8°14'11.0"S, 115°20'19.7"E	~1,400	2015	Wet-processed	Non-organic
Kopi Luwak	Bali Kintamani, Indonesia	8°14'11.0"S, 115°20'19.7"E	~1,400	2017	Wet-processed	Non-organic
Non-Jacu	Espírito Santo, Brazil	20°22'00.8"S, 41°02'59.3"W	~1,100	2016	Wet-processed	Organic
Non-Jacu	Espírito Santo, Brazil	20°22'00.8"S, 41°02'59.3"W	~1,100	2017	Wet-processed	Organic
Non-Jacu	Espírito Santo, Brazil	20°22'00.8"S, 41°02'59.3"W	~1,100	2017	Dry-processed	Organic
Jacu	Espírito Santo, Brazil	20°22'00.8"S, 41°02'59.3"W	~1,100	2016	Dry-processed	Organic
Jacu	Espírito Santo, Brazil	20°22'00.8"S, 41°02'59.3"W	~1,100	2017	Dry-processed	Organic

injector (Rheodyne), with a 20 μL loop and a L20314500010 Evaporative Light Scattering Detector (ELSD), set at 40°C, gain of 4 and nitrogen flow pressure of 350 kPa. The chromatographic separation was achieved by a C_{18} reverse-phase column (Zorbax NH_2, 250 × 4.6 mm, 5 μm, Agilent Technologies, Palo Alto, CA, USA). An isocratic elution during 10 min with acetonitrile and water (80:20) at a flow rate of 1.0 mL/min was used. Sucrose was quantified by external standardization using the commercial standard. The calibration curve ranged from 10 to 400 ppm and its coefficient of determination (R^2) was 0.9991. The method was previously validated by Perrone et al. (2008b).

Alkaloids: Caffeine and Trigonelline

The extraction of alkaloids was performed according to Perrone et al. (2008b), in triplicate. Chromatographic analysis was performed according to Alves et al. (2006). The LC system (Shimadzu, Kyoto, Japan) was the same as reported in item 2.5. The chromatographic separation was achieved with a C30 column (150 × 2.0 mm, 5 μm, Michrom BioResources, Auburn, USA) maintained at room temperature. The mobile phase used was a gradient among 0.3% aqueous formic acid (eluent A), methanol (eluent B), and acetonitrile (eluent C) with a flow rate of 0.7 mL/min. Before injection, the column was equilibrated with 95% A, 0% B, and 5% C during 40 min. Immediately after injection, this proportion was changed to 93% A, 0% B, and 7% C during 8 min and 87% A, 13% B, and 0% C until the end of the run in 10 min. Alkaloids were quantified by external standardization (absorbances at 260 nm and 272 nm for trigonelline and caffeine, respectively) using the commercial standards. The calibration curves for caffeine and trigonelline ranged from 0.5 to 20 ppm and their coefficients of determination (R^2) were 0.9992 and 0.9999, respectively. The method was previously validated by Perrone et al. (2008b).

Lipids

The following lipid classes were analyzed according to the method described by Novaes et al. (2018), with slight modifications: triacylglycerols (TAG), diacylglycerols (DAG), free fatty acids (FFA), sterols, diterpenes, and tocopherols. Extraction of lipids was performed in triplicate by adding an aliquot of 0.5 g of ground green coffee to 2 mL of hexane. The mixture was shaken by a magnetic bar at room temperature for 30 min. The suspension was filtered through a 0.22 μm cellulose ester membrane (Millipore, Brazil) and kept at −20°C until the GC-MS analysis.

The GC system (GC 6890, AgilentTechnologies, Palo Alto, CA, USA) comprised an automatic injector (7683) and a mass spectrometer (5975C). The chromatographic separation was achieved by a DB-17HT capillary column (50% phenyl and 50% methylsiloxane, 15 m × 0.25 mm i.d. × 0.15 μm, J&W Scientific, Agilent Technologies, Palo Alto, CA, USA). Helium was used as a carrier gas at a flow rate of 2 mL/min. The split ratio was 1:50, the injector was heated to 330°C, and a pressure pulse of 25 psi was applied during the initial 15 s. The oven temperature was raised from 50°C (0.25 min) to 380°C (10 min), at a heating rate of 15°C/ min. Mass spectrometer conditions were: ion source temperature at 230°C, quadrupole at 200°C, acceleration voltage of 200 eV, transfer line at 380°C, and ionization voltage of 70 eV. Mass spectra were obtained in scan mode (50–800 Da). Lipids were identified by comparison with NIST/EPA/NIH Mass Spectral Library databases (2014 version) and values ≥ 70% were accepted. When library spectra were not available, the identification was performed by mass spectral interpretation and comparison with profiles described in the literature from authentic standards, according to our previous works (Novaes et al., 2015, 2018, 2020). In order to quantify lipids, the same extracts were also analyzed in an Agilent 6850 GC instrument (Santa Clara, CA) equipped with a flame ionization detector (GC-FID), using the same chromatographic conditions described above, except for the carrier gas, which was hydrogen at a flow rate of 2 mL/min. FID was kept at 400°C. Lipids values obtained were normalized based on the total group peak areas according to Novaes et al. (2018). The method was previously validated by our group

and its performance parameters were reported by Novaes et al. (2015).

Statistical Analysis

Kopi Luwak coffee samples were subjected to principal component analysis (PCA) to investigate similarities among samples and possible associations of variables. PCA was performed using all individual non-volatile compounds, as well as using compounds grouped according to their chemical class (i.e., DAG, TAG, FFA, diterpenes, CGA, sterols). PCA analysis was performed by PAST for Windows (version 2.17c). Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Bioprocessing of Jacu and Kopi Luwak Coffee Beans Was Confirmed by Scanning Electron Micrography

Non-Jacu (wp) coffee beans showed fewer surface irregularities than non-Jacu (dp) coffee beans (**Figures 2A,B**). This characteristic may be related to damage of coffee beans cell membranes during the drying process (Borém et al., 2014), which may also cause minerals to leach, possibly leading to the lower ashes contents of non-Jacu (dp) (1.9 g/100 g) in comparison to non-Jacu (wp) (2.7 g/100 g) (**Table 2**).

All Jacu and Kopi Luwak coffee beans showed exfoliations on their surfaces (**Figures 2C–L**), similar to those observed by Marcone (2004) for different types of civet bioprocessed coffees. According to the author, these exfoliations are a consequence of the peristaltic movements of the animal's gastrointestinal tract during coffee digestion. Moreover, the acidic gastric juice and proteolytic digestive enzymes penetrate the coffee cherry endocarp leading to micro-pittings (Marcone, 2004). As a whole, micrographic results confirmed that Jacu and Kopi Luwak green coffee beans were bioprocessed.

Bioprocessing by the Jacu Bird Decreased Caffeine and CGA Contents and Modified Lipid Profile of Green Coffee

The main limitation of our study is the small number of samples analyzed, which is a consequence of the difficulty in obtaining coffee bioprocessed samples from reliable producers. Because of this, we did not had control over parameters such as post-harvesting processing and crop year and, consequently, could not perform statistical analyses to compare them. In that way, this study should be considered as a case study, but despite its limitations, it should be of interest for those working with coffee quality, especially bioprocessed samples, for which chemical composition data is scarce or inexistent.

The proximate composition of both Jacu coffee samples was according to the range reported in the literature for these components in non-bioprocessed *C. arabica* coffees (Clarke and Macrae, 1985). Bioprocessing by Jacu bird did not affect proteins, lipids, ashes, and dietary fibers contents of coffee beans (**Table 2**). Marcone (2004) and Mahendradatta et al. (2012) reported a decrease in protein contents of civet coffee, associated with both proteolysis and leaching during bioprocessing. However, mammals such as the civet "cat" present a larger gut size (greater total surface area and thus absorptive capacity), longer digesta retention time (Whorter et al., 2009), and higher pepsin activity in gastric juice (Reece et al., 2011) when compared to birds, which may explain our results in comparison to those previously reported for civet coffee. To compensate, birds seem to present a higher passive (paracellular) nutrient absorption than mammals (Whorter et al., 2009). Ashes and dietary fibers contents of our Brazilian (Espírito Santo) coffees from the 2016 and 2017 crops showed differences. Considering that these samples were from the same cultivars and harvested in the same farm, these differences are probably explained by the climate conditions in these years during coffee growth. According to the meteorology coordination of Espírito Santo state, Brazil (Incaper, 2022), the region where coffee samples were grown had higher minimum and maximum temperatures in 2016 than in 2017.

Caffeine contents in non-Jacu (both dp and wp) coffee samples were in accordance with the reported range for *C. arabica* non-bioprocessed coffees (0.6–1.8 g/100 g) (Cheng et al., 2016). Jacu coffees presented similar contents to that reported by Nishiguchi et al. (2017) for a civet sample (0.36 g/100 g). Bioprocessing caused a trend of reducing caffeine contents by about 69%, as observed by comparing non-Jacu (1.12 g/100 g, on average) and Jacu (0.34 g/100 g, on average) coffee samples (**Table 3**). There is some evidence in the literature that bioprocessed coffees show lower contents of caffeine than non-bioprocessed ones (Mahendradatta et al., 2012; Conti et al., 2013; Nishiguchi et al., 2017; Ifmalinda et al., 2019). Recently, Febrina et al. (2021) reported that civet coffee samples had lower caffeine contents (determined by NMR) than regular coffee. This work, however, is the first to analyze samples of bioprocessed and non-bioprocessed coffees from the same farmer (same harvest location and cultivar), which strengthens the relevance of this finding. Since caffeine is recognized as toxic for avians (Lightfoot and Yeager, 2008), it was probably not absorbed in the digestive tract of the Jacu bird. Baumann et al. (1995) reported that under simulated gastric conditions, caffeine was not released from guaraná (*Paullinia cupana*) seeds. Nevertheless, some authors suggest that caffeine could be fermented by gut microbiota (Mahendradatta et al., 2012; Ifmalinda et al., 2019), and many studies indicate that *Pseudomonas* spp. isolated from soil samples are able to metabolize caffeine (Mazzafera, 2002). These bacteria may also be found in the microbiota of birds, along with *Bacteroides*, *Clostridium*, *Lactobacillus*, *Streptococcus*, and *Campylobacter* (Hird et al., 2015), possibly explaining our results. The low caffeine content in Jacu coffee may impact its flavor, reducing its perceived strength, body, and bitterness (Clarke and Macrae, 1988; Cheng et al., 2016).

Trigonelline contents were similar between non-Jacu and Jacu coffee samples and in accordance with the range reported for *C. arabica* (0.3–1.3 g/100 g) (Cheng et al., 2016) (**Table 3**), suggesting that this alkaloid was more resistant to bioprocessing than caffeine during bird digestion. Also, there were no differences between non-Jacu and Jacu coffee samples concerning sucrose contents, which were in accordance with the range reported for *C. arabica* (5.0–12.0 g/100 g) (Redgwel and Fischer,

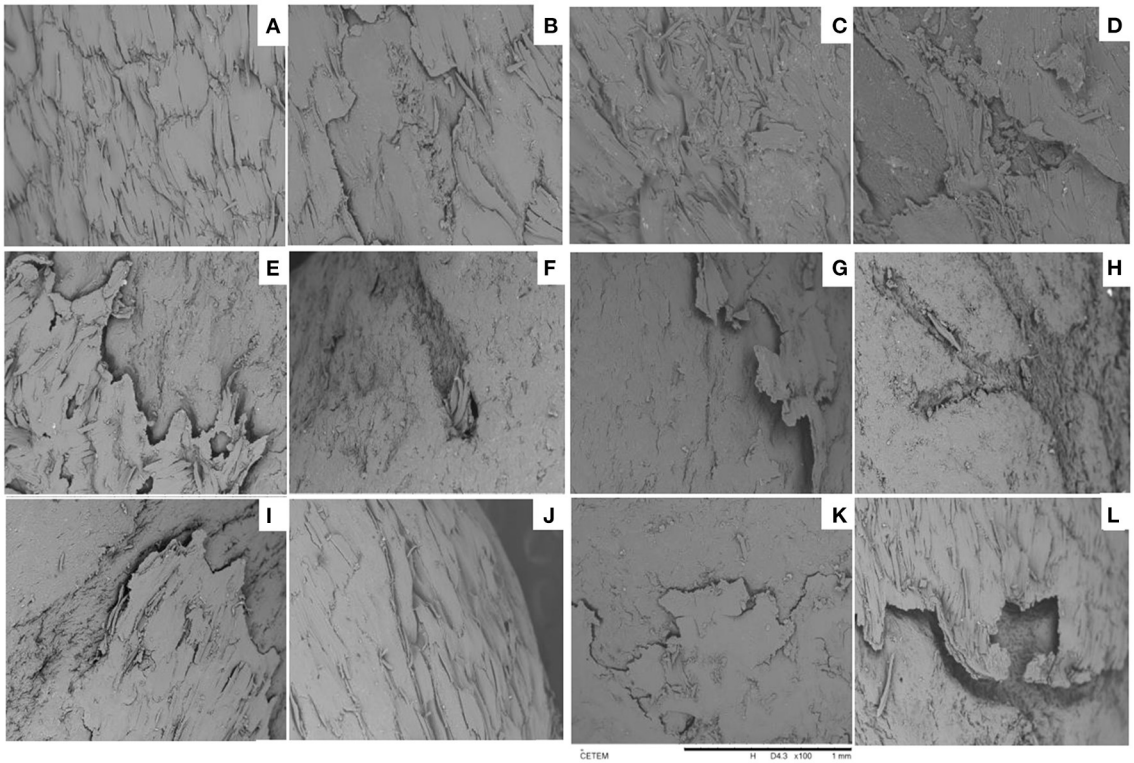

FIGURE 2 | Typical scanning electron micrographs of non-Jacu wet-processed crops 2016 and 2017 **(A)**, Non-Jacu dry-processed crop 2017 **(B)**, Jacu dry-processed crop 2016 **(C)**, Jacu dry-processed crop 2017 **(D)**, Sumatra Aceh Gayo dry-processed crop 2015 **(E)**, Sumatra Aceh Gayo dry-processed crop 2017 **(F)**, Sumatra Mandheling dry-processed crop 2015 **(G)**, Sumatra Mandheling dry-processed crop 2017 **(H)**, East Java wet-processed crop 2015 **(I)**, Central Java wet-processed crop 2017 **(J)**, Bali Kintamani wet-processed crop 2015 **(K)**, and Bali Kintamani wet-processed crop 2017 **(L)** coffee samples.

TABLE 2 | Proximate composition (g/100 g dry weight) of non-Jacu, Jacu and Kopi Luwak coffee samples[a].

Sample	Proteins	Lipids	Ashes	Dietary fibers	Carbohydrates
Brazilian Jacu coffees					
Non-Jacu wet-processed crop 2016	13.0	10.0	3.7	35.4	38.3
Non-Jacu wet-processed crop 2017	11.7	9.3	2.7	56.2	20.3
Non-Jacu dry-processed crop 2017	11.1	11.6	1.9	53.2	22.2
Jacu dry-processed crop 2016	12.1	11.8	3.8	45.5	27.3
Jacu dry-processed crop 2017	11.3	12.2	2.7	47.9	26.1
Indonesian Kopi Luwak coffees					
Sumatra Aceh Gayo dry-processed crop 2015	11.8	9.4	4.0	65.1	9.9
Sumatra Aceh Gayo dry-processed crop 2017	13.2	13.7	5.3	51.4	16.0
Sumatra Mandheling dry-processed crop 2015	11.9	10.1	4.0	59.0	15.0
Sumatra Mandheling dry-processed crop 2017	12.8	14.3	5.2	53.4	14.4
East Java wet-processed crop 2015	13.3	9.7	4.2	61.4	11.4
Central Java wet-processed crop 2017	13.7	13.4	5.3	61.1	5.6
Bali Kintamani wet-processed crop 2015	12.2	9.8	4.3	48.5	25.3
Bali Kintamani wet-processed crop 2017	13.6	11.9	5.5	54.8	13.5

[a] *Results are presented as means of three analytical replicates; coefficient of variation was lower than 10% for all coffee samples.*

2006). Sucrose contents were also not affected by post-harvesting processing (dry vs. wet-processing). Still, differences were observed between 2016 and 2017 crops, possibly related to the climate conditions in these years during coffee growth. Insolation intensity, for instance, is positively correlated to sucrose biosynthesis (Cheng et al., 2016).

TABLE 3 | Caffeine, trigonelline, sucrose and chlorogenic acids contents (g/100 g dry weight) in non-Jacu, Jacu and Kopi Luwak coffee samples[a].

Sample	Caffeine	Trigonelline	Sucrose	Chlorogenic acids				
				CQA	FQA	diCQA	*p*-CoQA	Total
Brazilian Jacu coffees								
Non-Jacu wet-processed crop 2016	1.07	0.99	11.44	3.70	0.26	0.28	0.07	4.30
Non-Jacu wet-processed crop 2017	1.12	1.03	7.17	3.44	0.17	0.36	0.03	3.99
Non-Jacu dry-processed crop 2017	1.16	1.00	7.60	3.73	0.20	0.41	0.06	4.38
Jacu dry-processed crop 2016	0.35	0.94	11.50	2.86	0.16	0.22	0.03	3.28
Jacu dry-processed crop 2017	0.34	1.14	6.71	3.59	0.03	0.19	0.01	3.82
Indonesian Kopi Luwak coffees								
Sumatra Aceh Gayo dry-processed crop 2015	0.47	1.04	6.42	2.85	0.18	0.33	0.1	3.45
Sumatra Aceh Gayo dry-processed crop 2017	0.46	1.05	10.92	5.67	0.26	0.71	ND[b]	6.64
Sumatra Mandheling dry-processed crop 2015	0.36	1.20	3.99	3.07	0.20	0.30	0.02	3.58
Sumatra Mandheling dry-processed crop 2017	0.53	1.20	11.43	5.73	0.26	0.60	0.005	6.60
East Java wet-processed crop 2015	0.59	1.16	4.99	2.67	0.15	0.18	0.1	3.10
Central Java wet-processed crop 2017	0.56	1.18	9.40	5.67	0.25	0.64	0.009	6.57
Bali Kintamani wet-processed crop 2015	0.45	1.01	6.42	2.53	0.16	0.23	0.04	2.97
Bali Kintamani wet-processed crop 2017	0.47	1.01	12.84	6.44	0.32	1.07	0.008	7.84

[a] *Results are presented as means of three analytical replicates; coefficient of variation was lower than 10% for all coffee samples. CQA, Caffeoylquinic acids; FQA, feruloylquinic acids; diCQA, dicaffeoylquinic acids; p-CoQA, coumaroylquinic acids.*
[b] *Not detected (below 0.1 ppm for CGA).*

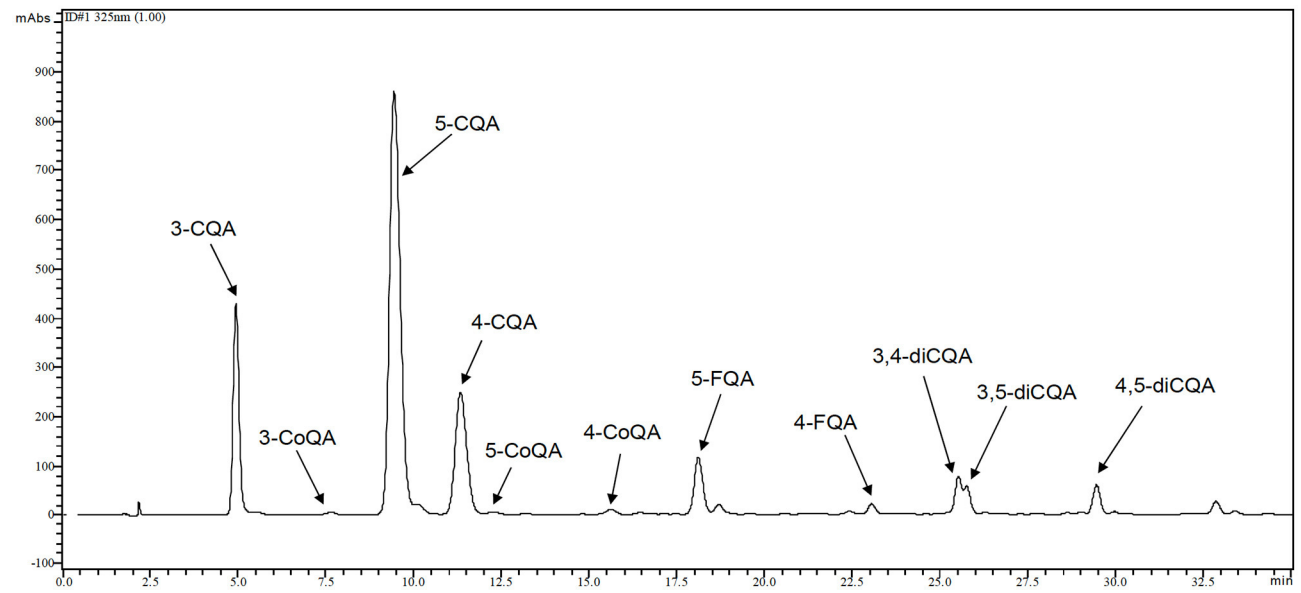

FIGURE 3 | Typical chromatographic separation of chlorogenic acids (CGA) released from non-Jacu, Jacu and Kopi Luwak coffee samples. CQA, Caffeoylquinic acid; FQA, feruloylquinic acid; diCQA, dicaffeoylquinic acid; *p*-CoQA, coumaroylquinic acid.

Eleven CGA were quantified in non-Jacu and Jacu coffee samples: 3-CQA, 4-CQA, 5-CQA, 3-CoQA, 4-CoQA, 5-CoQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA (**Figure 3**). CQA was a major class (86.2%), followed by di-CQA (7.9%), FQA (4.9%), and *p*-CoQA (0.9%) (**Table 3**). CGA contents of non-Jacu samples (4.2 g/100 g, on average) were close to the lower limit of the reported range for *C. arabica* in the literature (Farah and Donangelo, 2006; Cheng et al., 2016) (4.0–8.4 g/100 g) and CGA contents of Jacu coffee samples (3.4 g/100 g, on average) were similar to that reported by Cheong et al. (2013) for Kopi Luwak coffee (3.0 g/100 g). Bioprocessed samples seem to contain less CGA than non-bioprocessed ones, and this difference could be possibly explained by the degradation and/or absorption of these compounds during the bird's digestion. When CGA classes are considered separately, similar behaviors could be noticed. Even though it is well known

that CGA is absorbed and extensively metabolized in humans (Clifford et al., 2020) and rodents (Clifford, 2000), there are no studies investigating these processes in birds. Nevertheless, Karasov et al. (2012) reported that birds have a higher capacity of absorbing water-soluble secondary plant metabolites, such as phenolic compounds, than rodents. Moreover, Zhang et al. (2020) recently showed that CGA consumption minimizes damage to the small intestine structure of chickens, as well as improves antioxidant capacity and inhibits the transcriptional activity of inflammatory cytokines, implying CGA absorption. However, further studies with adequate statistical power must be conducted to confirm these preliminary observations.

The lipid fraction of all green coffee samples was composed of FFA, diterpenes, tocopherols, sterols, DAG, and TAG (**Figure 4**), as reported in the literature for *C. arabica* non-bioprocessed green coffees (Speer and Kölling-Speer, 2006; Farah, 2012; Novaes et al., 2018). TAG were the predominant class, representing, on average, 88.7% of lipids (**Figure 5**). Dipalmitoyl-linoleoyl glycerol (PPL) and palmitoyl-dilinoleoyl glycerol (PLL) were those found in the highest proportions, on average of 32.7 and 27.6%, respectively, similar to those reported by González et al. (2001). Other seven TAG were found in coffee samples at lower proportions: palmitoyl-stearoyl-linoleoyl glycerol (PSL), palmitoyl-oleoyl-linoleoyl glycerol (POL), palmitoyl-linoleoyl-arachidonoyl glycerol (PLA), stearoyl-oleoyl-linoleoyl glycerol (SOL), distearoyl-linolenyl glycerol (SSLn), oleoyl-linoleoyl-linolenyl glycerol (OLLn) and dilinoleoyl-linolenyl glycerol (LLLn). FFA accounted for an average of 1.7% of lipids (**Figure 5**), similar to that reported by Farah (2012). Linoleic, oleic, and palmitic acids corresponded, on average, to 38.7%, 33.1%, and 28.2% of this lipid class, respectively, in accordance with previous reports in the literature (Speer and Kölling-Speer, 2006; Oliveira et al., 2014). Campesterol, β-sitosterol, and stigmasterol were the sterols identified in the coffee samples (**Figure 6**), accounting for 4.8% of total lipids, in accordance with Novaes et al. (2015). β-Sitosterol was the most abundant sterol, representing on average 50.7% of this lipid fraction, similar to that reported by Farah (2012).

Kahweol and cafestol were the two diterpenes observed in all samples (**Figure 6**), as previously reported by the literature (Clifford, 1985; Clarke and Macrae, 1988; Tinoco et al., 2019; Cyrus et al., 2021). In their free forms (dialcohol), these compounds together accounted for an average of 0.9% of lipids, similarly to that reported by Speer and Kölling-Speer (2006). As diterpenic fatty acids, kahweol palmitate and cafestol palmitate were identified in all samples, as previously reported by the literature (Kurzrock and Speer, 2001; Speer and Kölling-Speer, 2006; Lima et al., 2020). The most abundant was cafestol palmitate, which represented on average 66% of this class, and together these compounds accounted from 0.3 to 4.1% of lipids, on average. The literature reports a wide range for the contents of esterified diterpenes in *C. arabica* coffee oil: from 1.1 to 18.0% (Speer and Kölling-Speer, 2006; Novaes et al., 2015, 2020). The ratio between cafestol and kahweol, which has been suggested as an indicator of cup quality (Novaes et al., 2015), ranged from 0.5 to 2, in accordance with the literature (Gross et al., 1997; Silva et al., 2012; Oliveira et al., 2014).

Bioprocessing by the Jacu bird seems to have affected the lipid profile of coffee samples, especially the contents of TAG. While PPA, LLLn and PPL seem to have decreased, SOL, POL and OLLn seem to have increased, and PSL, PLL and SSLn did not change. DAG contents were not modified due to bioprocessing. In avians, lipids are digested through hydrolysis by lipolytic enzymes (colipase and pancreatic lipase) followed by absorption in the small intestine (Bauer et al., 2005). Together, our results suggest that TAG containing two saturated fatty acids (PPL and PLA) were preferably hydrolyzed in detriment to those containing two unsaturated fatty acids (POL, SOL, and PLL) and that the FFA produced were absorbed in the digestive tract of the Jacu bird. Alternatively, FFA could have been degraded by the avian gut microbiota (e.g., *Bacteroides*, *Clostridium*, *Lactobacillus*, *Streptococcus*, and *Campylobacte*). Bioprocessing by the Jacu bird seems to have also increased campesterol contents, possibly associated to a higher absorption of the other two sterols. Bioprocessing did not modify diterpenes proportions, consequently not changing the ratio between cafestol and kahweol, an indicator of cup quality (Novaes et al., 2015).

Post-harvest processing of the Brazilian coffee samples also affected their lipid profile. Wet-processed samples seem to have higher contents of PLA, LLLn, stigmasterol, β-sitosterol and β-tocopherol than dry-processed ones. Differently from most enzymes, which need water activities above 0.7 to catalyze reactions, lipases have a unique ability to be active at lower water activities (Wehtje and Adlercreutz, 1997). Therefore, the observed changes in lipids could be associated to different lipase activities in dry and wet-processed samples. Moreover, each post-harvest process entails a different fermentation step, with different microbiota, which may also have influenced their lipid profile.

Crop Year Rather Than Location or Post-harvest Processing Discriminated Kopi Luwak Coffee Samples

The average contents of proteins (12.8 g/100 g dwb), lipids (11.5 g/100 g dwb), ashes (4.7 g/100 g dwb) and total carbohydrates (70.7 g/100 g dwb) in Kopi Luwak coffee samples (**Table 2**) were similar to those previously reported in the literature (Marcone, 2004; Muzaifa and Hasni, 2016; Hadipernata and Nugraha, 2018; Muzaifa et al., 2020), with the exception of the lipid contents reported by Muzaifa and Hasni (2016) (on average 1.3 g/100 g), which was much lower than expected for green coffee beans. This study is the first to report the dietary fiber contents in Kopi Luwak coffee samples (on average 56.8 g/100 g dwb) (**Table 2**), which were slightly higher than the 40–50% content expected to be found in green coffee beans (Trugo, 1985), possibly due to the relative decrease of digestible nutrients. Kopi Luwak samples from the 2015 crop, independently of different harvesting regions and post-harvest processes, had lower contents of proteins (8%), lipids (27%) and ashes (23%) compared with those from the 2017 crop, probably due to different weather conditions in these years.

Kopi Luwak coffee samples had an average caffeine content of 0.48 g/100 g (dwb) (**Table 3**), which is similar to that reported

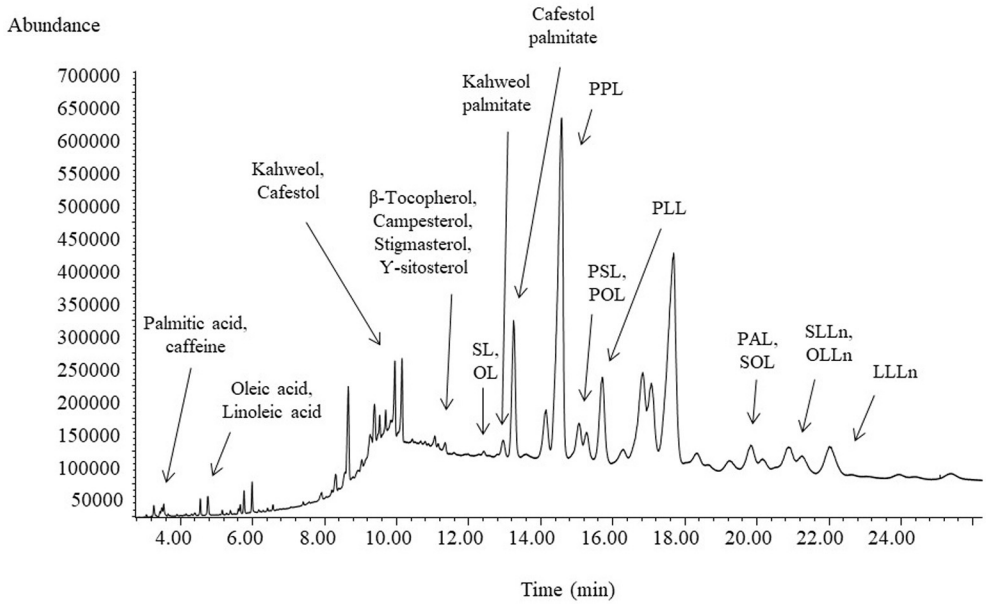

FIGURE 4 | Typical chromatogram of the crude lipid fraction from Kopi Luwak coffee samples. Dashed lines indicate regions of fatty acids, caffeine, free diterpenes, tocopherols and sterols, diacylglycerols, esterified diterpenes and triacylglycerols. P, palmitic; O, oleic; L, linoleic; S, stearic; A, arachidonic; Ln, linoleic.

by Nishiguchi et al. (2017) (0.36 g/100 g) for Kopi Luwak and that found for Jacu coffees in the present study (0.34 g/100 g, on average), but lower than that reported by Muzaifa et al. (2020) (on average 1.20 g/100 g) for Kopi Luwak and that of non-bioprocessed green *C. arabica* samples (0.6–1.8 g/100 g) (Cheng et al., 2016). This result reinforces the previously mentioned hypothesis that bioprocessing reduces green coffee caffeine content. Trigonelline average content in Kopi Luwak coffee samples was 1.11 g/100 g (dwb) (**Table 3**), which is in accordance with the content reported for non-bioprocessed green *C. arabica* samples (0.3–1.3 g/100 g) (Cheng et al., 2016). To the best of our knowledge, our study is the first to analyze this compound in Kopi Luwak coffee. Sucrose contents showed a wide variation among Kopi Luwak coffee samples, ranging from 3.99 to 12.84 g/100 g (dwb) (**Table 3**), in accordance with the literature for non-bioprocessed green *C. arabica* (Redgwel and Fischer, 2006). While samples from the 2015 crop (on average, 5.5 g/100 g dwb) had sucrose contents similar to that reported by Muzaifa (2018) for Kopi Luwak coffees (on average, 6.3 g/100 g), samples from the 2017 crop had twice as much (on average, 11.1 g/100 g dwb) ($p < 0.0001$). This difference may be explained by insolation intensity in these years, as shading negatively affects sucrose biosynthesis (Cheng et al., 2016).

The same CGA found in non-Jacu and Jacu coffee samples were observed in Kopi Luwak coffees, with the same order of abundance for CGA classes: CQA (80.0%), di-CQA (8.7%), FQA (4.3%) and *p*-CoQA (1.1%) (**Table 3**). 5-CQA was also the most abundant, representing an average of 62.2% of the total CGA. Kopi Luwak samples had an average content of total CGA of 5.09 g/100 g (dwb), with samples from the 2017 crop showing twice as much CGA (on average, 6.91 g/100 g dwb) than those from the 2015 crop (on average, 3.28 g/100 g dwb), a behavior observed for all CGA classes. Therefore, while Kopi Luwak samples from 2015 had total CGA contents very similar to those reported by Muzaifa et al. (2020) for Kopi Luwak (on average 3.73 g/100 g), samples from 2017 were more similar with non-bioprocessed *C. arabica* (Farah and Donangelo, 2006; Cheng et al., 2016), which usually contains more than 5% of total CGA.

Similar to Jacu coffee samples, TAG were the predominant class of lipids in Kopi Luwak coffee samples (83.3% of lipids, on average), with PPL and PLL as the major ones (26.5 and 21.9%, respectively, on average) (**Figure 5**). On the other hand, Kopi Luwak coffee samples had higher FFA contents (2.69%) and lower DGA contents (0.30%) than Jacu coffee samples (1.26 and 0.54%, respectively). Kopi Luwak coffee samples also showed lower content of sterols (1.70%) and higher content of diterpenes (10.97%) when compared to Jacu coffee samples (4.57 and 2.76%, respectively) (**Figure 6**). These results may be related to differences in lipids digestion process of mammals and avians.

In order to understand the contribution of volatile precursors in the distinction of each Kopi Luwak coffee sample and their relationship with crop year, post-harvesting processing and growing conditions, the principal component analysis (PCA) was performed, either grouping compounds according to their chemical class (**Figure 7A**) or considering each individual compound as a separate variable (**Figure 7B**). The first three principal components accounted together for 73.7 and 66.4% of the variance in these plots, respectively. In both plots, samples harvested in 2015 are located in the left quadrants, whereas those from 2017 are in the right

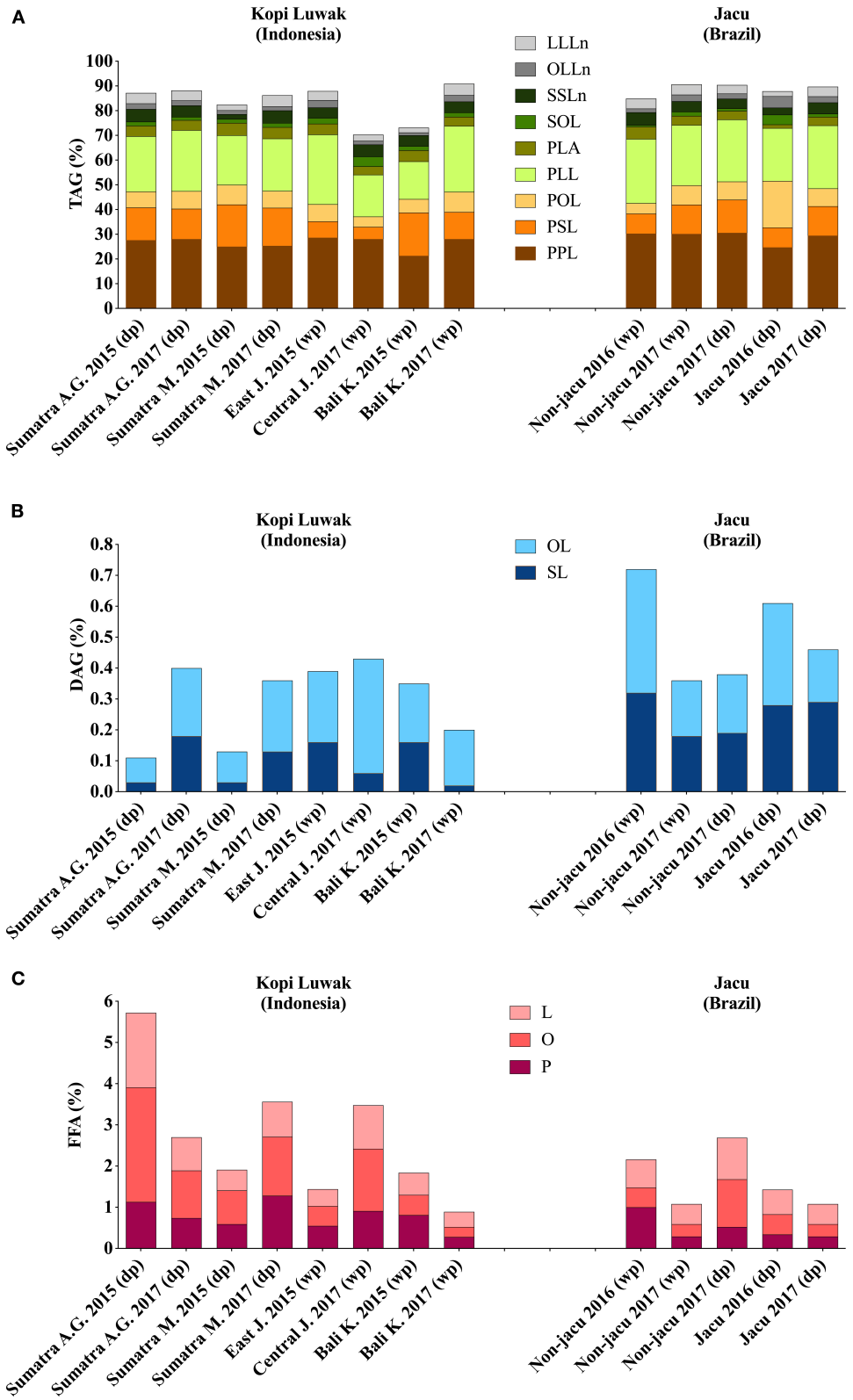

FIGURE 5 | Profile of triacylglycerols [TAG, **(A)**], diacylglycerols [DAG, **(B)**] and free fatty acids [FFA, **(C)**] of coffees from Indonesia (Kopi Luwak) and Brazil (Non-jacu and Jacu). Indonesian samples were from 2015 and 2017 crops of different regions (A.G., Aceh Gayo; M., Mandheling; J., Java; K., Kintamani). Brazilian samples were from 2016 and 2017 crops. Samples were either dry processed (dp) or wet processed (wp). P, palmitic; O, oleic; L, linoleic; S, stearic; A, arachidonic; Ln, linoleic.

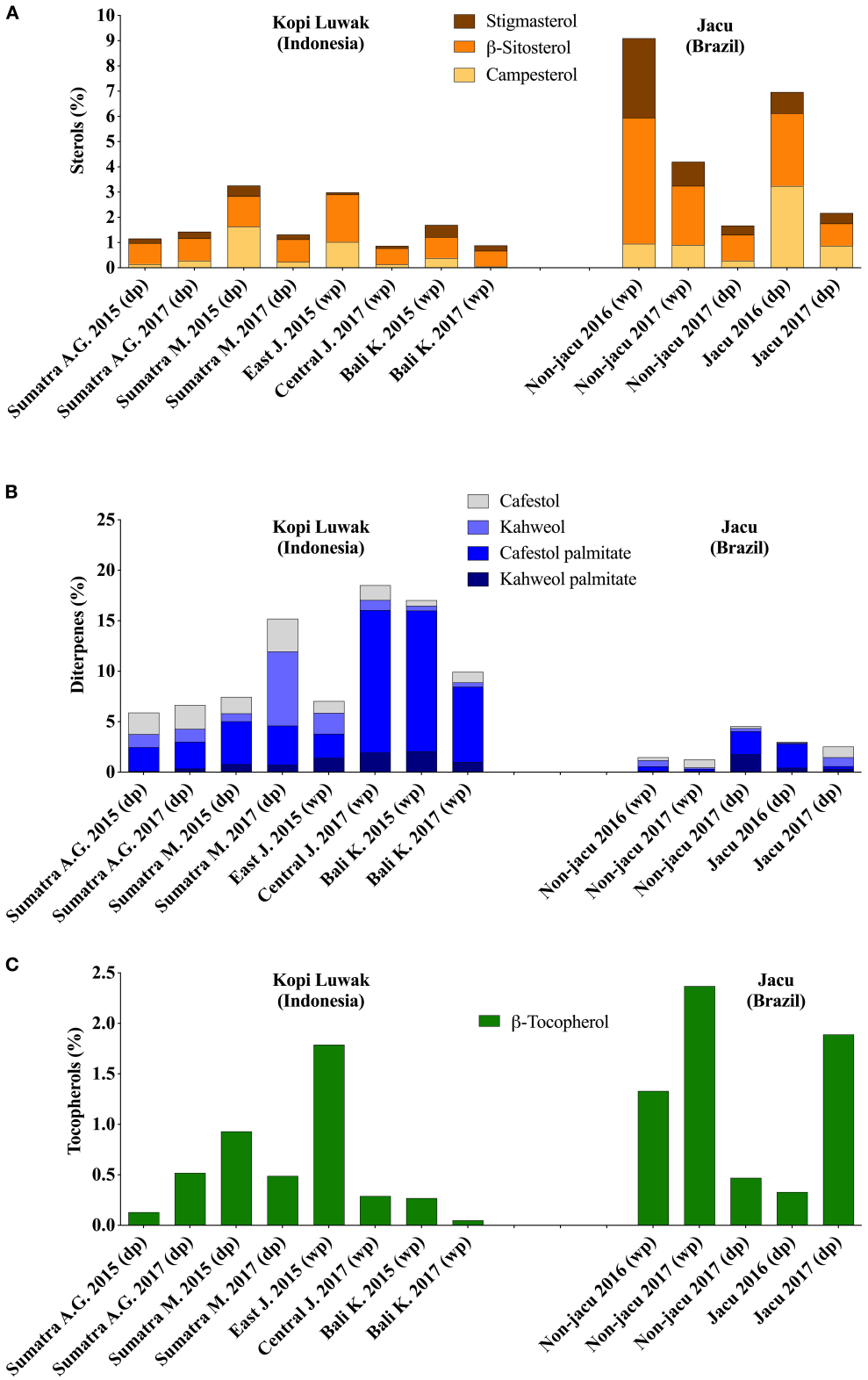

FIGURE 6 | Profile of sterols **(A)**, diterpenes **(B)**, and tocopherols **(C)** of coffees from Indonesia (Kopi Luwak) and Brazil (Non-jacu and Jacu). Indonesian samples were from 2015 and 2017 crops of different regions (A.G., Aceh Gayo; M., Mandheling; J., Java; K., Kintamani). Brazilian samples were from 2016 and 2017 crops. Samples were either dry processed (dp) or wet processed (wp).

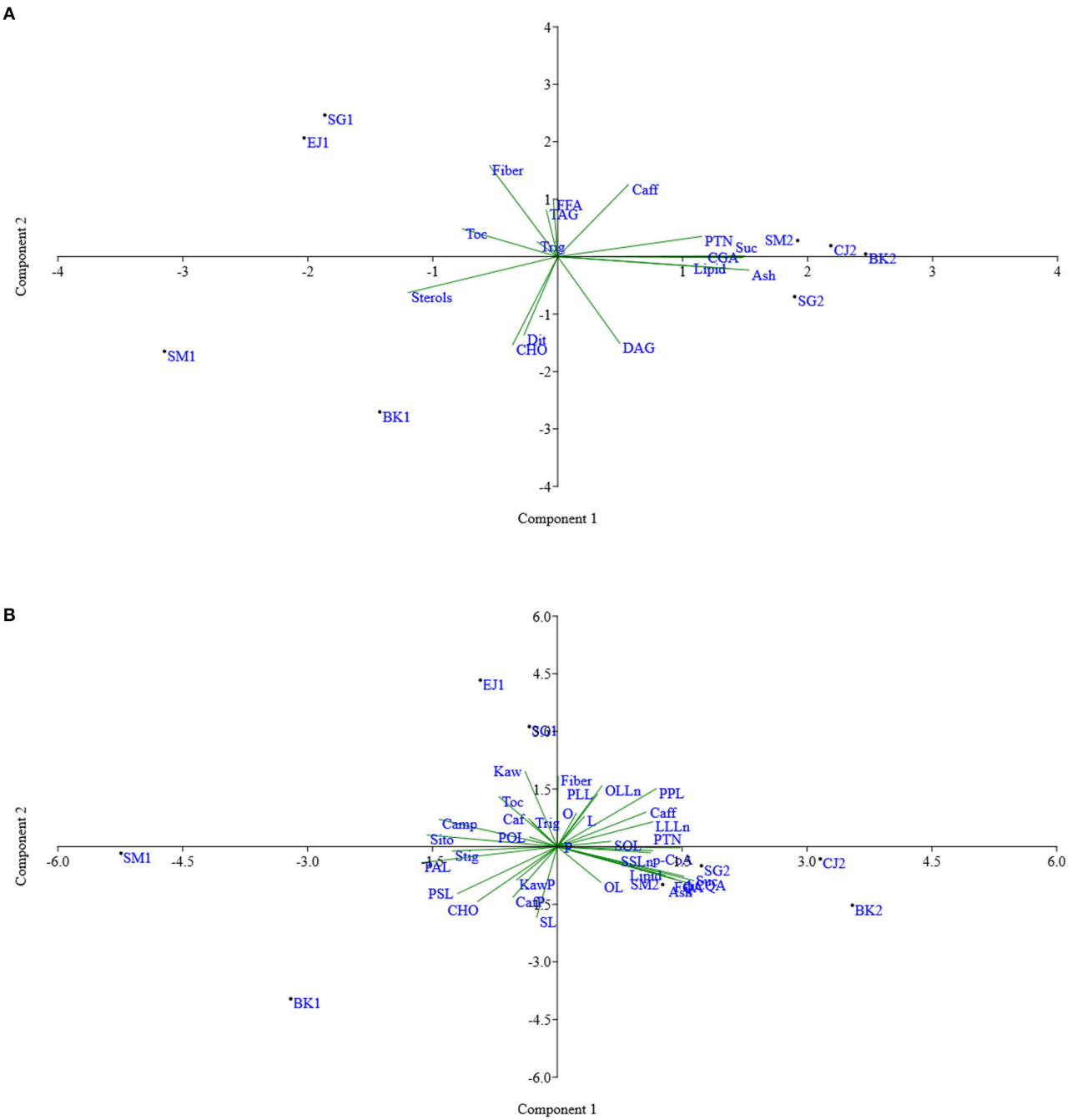

FIGURE 7 | Principal component analysis (PCA) concerning the distribution of grouped compounds of Kopi Luwak (SG, Sumatra Aceh Gayo; SM, Sumatra Mandheling; EJ, East Java; CJ, Central Java; BK, Bali Kintamani) coffee samples from crops 1 (2015) and 2 (2017) **(A)** or in relation to all measured individual **(B)**. PTN, Protein; CHO, Carbohydrates; Caff, Caffeine; Trig, Trigonelline; Suc, Sucrose; Dit, Diterpenes; CGA, Chlorogenic acids; FFA, Free fatty acids; DAG, Diacylglycerols; TAG, Triacylglycerols; P, palmitic; O, oleic; L, linoleic; S, stearic; A, arachidonic; Ln, linolenic; Kaw, Kaweol; Caf, Cafestol; Toc, β-Tocopherol; Camp, Campesterol; Sito, γ-Sitosterol; Stig, Stigmaesterol; KawP, Kahweol palmitate; CafP, Cafestol palmitate.

ones, indicating that the crop year, in other words weather conditions, was a contributor to discriminating samples. The most important contributors for the discrimination of these samples were total lipids, ashes, total CGA, sucrose and proteins, all of which were found at higher contents in 2017 samples compared to 2015 ones. Neither the harvesting location nor the post-harvest processing have discriminated Kopi Luwak, as samples with equivalent characteristics (SG and BK, for instance) were located in opposite quadrants of the PCA plots.

CONCLUSIONS

This was the first study to perform a detailed chemical characterization of exotic coffees, with emphasis on flavor precursors. Moreover, the effect of bioprocessing on these components was evaluated for the first time, specifically for Jacu coffee. The digestion process by the Jacu bird seem to have modified the profile of several flavor precursors, namely caffeine, CGA and TAG. However, further studies with adequate statistical power are needed to confirm these preliminary observations. The crop year of Kopi Luwak affected the flavor precursors composition, namely total lipids, total CGA, sucrose and proteins. However, it was not possible to identify which specific climatic conditions of these crop years led to these differences. All these changes may affect the formation of volatile compounds upon roasting, thus contributing to the unique flavor and aroma of these exotic coffees.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

BR: methodology, validation, formal analysis, investigation, data curation, and writing—original draft. MB: formal analysis and investigation. FN: methodology, validation, data curation, and writing—review and editing. MG, DF, and CR: resources and writing—review and editing. JN: conceptualization, data curation, writing—review and editing, supervision, project administration, and funding. DP: conceptualization, data curation, writing—review and editing, visualization, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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