Morpho-physiological changes during leaf development of robusta coffee under shade condition

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ABSTRACT

Background and Objective: Robusta coffee cultivars, local and Chumphon 2, are commonly grown in mixed orchards in Thailand. However, there is a lack of data on the stages of leaf development under shaded conditions. This research aims to monitor the morpho-physiological changes in leaves during leaf development of these two coffee cultivars.

Methodology: One-year-old Robusta coffee plants of two cultivars were planted in plastic baskets and grown under 80% shade in a greenhouse. Leaves that began unfolding and measured 4–7 cm in length (considered 0 days old) were selected, with one leaf per plant for ten plants per cultivar. The leaf size, leaf greenness (SPAD units), net photosynthetic rate (*Pn*), and dark respiration rate (*Rd*) were monitored daily using LI-6400 (LI-COR, USA) until all parameters stabilized. The data were analyzed using RStudio software (package 'ggplot2').

Main Results: The leaves of both coffee cultivars took 28–30 days to reach full maturity, with development divided into five stages. At 0 days old (stage 1), the leaves unfold. At 7 days old (stage 2), the leaves grow and expand to half their maximum size (52.23 cm^2). Leaf greenness and *Pn* increase rapidly, while *Rd* decreases quickly. At 14 days old (stage 3), the leaves reach their maximum *Pn* (3.32 $\mu mol\ m^{-2}\ s^{-1}$). Leaf expansion continues rapidly, with a slight reduction in leaf greenness. *Rd* continues to decrease steadily. At 21 days old (stage 4), the leaves reach full size (104.46 cm^2). Leaf greenness increases, *Pn* stabilizes, and *Rd* decreases slightly. At 28 days old (stage 5), *Rd* reaches its lowest point and stabilizes (0.34 $\mu mol\ m^{-2}\ s^{-1}$). Leaf greenness increases, leaf size stabilizes, and *Pn* remains constant.

Conclusions: The development of the leaves of the two Robusta coffee cultivars can be divided into five stages: 1) Leaves pair unfolding, 2) Leaves expand to half their maximum size, 3) *Pn* reaches its peak value, 4) Leaves reach their maximum size, and 5) *Rd* stabilizes.

Keywords: Photosynthesis rate, respiration rate, leaf area, leaf greenness, leaf age

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INTRODUCTION

Coffee is an important agricultural product and a popular beverage in Thailand, and it is among the top 10 global producers (Jongwutiwes, 2020). While Robusta coffee has traditionally been used for industrial purposes, there is a growing appreciation for specialty Arabica and high-quality Robusta varieties (Hetzel, 2011). Arabica is mainly cultivated in northern Thailand, while Robusta is grown in the southern regions. Recently, Robusta plantations have expanded to the north and northeast, where they are intercropped with bananas, avocados, and macadamia trees to provide shade, thereby promoting sustainability (Office of Agricultural Economics, 2020). Thailand has developed specific Robusta cultivars that are well-suited to local conditions. For example, Chumphon 2, a variety developed by the Chumphon Horticultural Research Center, is known for its robustness and adaptability due to its Congolese heritage (Lertwatthanakiat *et al.*, 2024). In addition, local strains have been identified and propagated for their unique suitability to specific microclimates and farming practices (Musigapong *et al.*, 2022).

Environmental factors, such as light exposure, play a crucial role in the growth and development of coffee plants. Research has shown that Robusta coffee cv. Chumphon 2 grown in shaded environments has different physiological characteristics than those grown in full sunlight. For example, plants grown in shade have lower specific leaf weight, smaller stomatal size, reduced photosynthetic rate, and decreased water use efficiency (Yanpisitkun *et al.*, 2019). The position of the leaf nodes (1st, 3rd, and 5th) also significantly affects these physiological responses and leaf characteristics (Yanpisitkun *et al.*, 2019). Shaded cultivation of Robusta coffee affects stem and canopy growth, resulting in larger leaf areas and fewer nodes (DaMatta, 2004; Jaramillo-Botero *et al.*, 2010). This type of cultivation induces various morphological and physiological responses, such as changes in leaf morphology, stomatal density and size, and photosynthesis and transpiration efficiency (Morais *et al.*, 2004; Franks *et al.*, 2009; Restrepo-Díaz *et al.*, 2010). Environmental conditions and seasons can also influence these adaptations (Kufa and Burkhardt, 2011). The study of leaf age dynamics and the cultivation of shaded Robusta coffee cultivars can provide valuable insights for agricultural practices, such as optimizing planting density, adjusting canopy management techniques, and selecting appropriate shading strategies. It is essential to ensure that Robusta coffee plants receive sufficient light for optimal photosynthesis, allowing for integrated and effective light management in coffee cultivation. This research is focused on examining the morphological and physiological changes during leaf development under shaded conditions in two different cultivars, Chumphon 2 and a local Thai variety, of young Robusta coffee plants.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Two cultivars of Robusta coffee plantlets, namely Chumphon 2 (CH2; *Coffea canephora* Pierre ex A. Froehner cv. Chumphon 2) and a local Thai variety (LO; the Robusta coffee grown in Thailand, belonging to the species *Coffea canephora*, often selected or bred for traits well-suited to the local environment, such as disease resistance, yield, and quality), were selected for an experimental study at the agricultural field of Kasetsart University in Bangkok, located at 13°85' North and 100°56' East, with an elevation of 1 meter above sea level, from January 2023 to May 2024. The experiment was conducted in nurseries (4 m × 9 m × 3 m) covered with black nylon nets that restricted 80% of the light transmission over the top and pulled the sides down halfway at a 45-degree angle on all four sides. Environmental data were collected every 30 min during the study. A data logger recorded air temperature and relative humidity (EasyLog EL-USB, Lascar Electronics Ltd., PA, USA). At the same time, light intensity was measured by line quantum sensors (LI-191, LI-COR Biosciences, Inc., Lincoln, NE, USA) with data loggers (Li-1400, LI-COR Biosciences, Inc., Lincoln, NE, USA). At the experimental site, the average day/night temperature and relative humidity were maintained at 31 ± 4/28 ± 2°C and 69 ± 11/76 ± 8%, respectively. The day length was 12 ± 0.5 hours (from 6:00−18:00), and the maximum light intensity ranged from 224−333 μmol $m^{-2} s^{-1}$.

Ten twelve-month-old coffee plants with approximately eight pairs of fully expanded leaves per cultivar were transplanted into 16-liter plastic baskets (26 cm in height × 36 cm in diameter), with the plants ranging in height from 60 to 80 cm. The baskets were filled with a growing soil medium, well-decomposed cow dung, and rice husk in a

6:1:2 ratio (v/v). Throughout the experimental period, chemical top-dressing fertilization was conducted using the NPK 16-16-16 formula, with 2 g per basket applied every 2 weeks at each fertilization session, a total of 34 times. Weeds were controlled manually, and the plants were watered daily to maintain the substrate at field capacity.

Sampling and Measurement

Sampling

The observation was conducted 12 months after transplanting, following the formation of visible primary branches. Newly emitted unfolding leaves, about 4–7 cm in length, on plagiotropic branches for each plant and cultivar were monitored, with ten randomly selected leaves (n = 10) considered day 0. Morphological and physiological changes were observed on the same leaves from day 0, and all parameters were monitored every day until they stabilized.

Morphological changes monitoring

Photographs were taken with a digital camera (PowerShot G7X Mark III, Canon Inc., Tokyo, Japan) until full leaf expansion. Images were always captured between 9 and 10 A.M. These photos were scaled and analyzed using Image J (http://rsb.info.nih.gov/ij/) according to Koyama (2023). Leaf greenness (SPAD units) was also measured using a chlorophyll meter (SPAD-502 Plus, Minolta, Tokyo, Japan). Each leaf sample was randomly measured at five positions around the leaf blade, and each leaf's average SPAD value was determined. The main changes in leaf color, shape, and size were identified, and the complete leaf development reference stages were defined.

Physiological changes monitoring

Photosynthesis rate (*Pn*) and dark respiration rate (*Rd*) were measured using a portable photosynthesis system (LI-6400XT, LI-COR Biosciences, Inc., Lincoln, NE, USA) equipped with a 2 × 3 cm broadleaf chamber and an integrated light source (LI-6400-02B, LI-COR Biosciences, Inc., Lincoln, NE, USA). Measurements were performed at a flow rate of 500 cm^3/min, with a CO_2 concentration of 400 µmol mol^{-1} (controlled by a CO_2 cylinder) and relative humidity ranging from 60–70%. Leaf temperature ranged from 30 to 35°C, and photosynthetic photon flux (PPF) was set at 800 µmol m^{-2} s^{-1} for *Pn* measurements and 0 µmol m^{-2} s^{-1} for *Rd* measurements. Measurements were made between 09:00 and 15:00 hours and recorded when the total coefficient of variation (CV) was less than 0.1%.

Estimation of the leaf development phase

The average leaf area, *Pn*, and *Rd* parameters of all Robusta coffee cultivars were used daily to determine the leaf development stages. This method, modified from Sesták (2012), identifies the periods as follows: the period to reach half of the maximum leaf area, photosynthetic maturity, maximum leaf area expansion, and the leaf mature non-senescent stage.

Statistical Analysis

Since the leaf area, greenness, *Pn*, and *Rd* curves are nonlinear, the curve was fitted to the scatter plot to assess, using the day after leaf unfolding as a predictor. To assess the variability in response to leaf stage development, linear regressions were applied using leaf area, leaf greenness, *Pn*, and *Rd* as response variables and the day after leaf unfolding as a separate predictor for each leaf stage development. Then, the conditional (R^2) coefficient of determination was computed to assess the amount of variance explained. Correlation matrices were determined between dependent variables (leaf area, leaf greenness, *Pn*, and *Rd*). All analyses were performed using RStudio v. 2024.04.1+748 (RStudio Team, 2022). Plots were made using the package ggplot2 (Wickham, 2016). Correlation matrices between dependent variables were determined with the "ggpubr" package (https://github.com/kassambara/ggpubr).

RESULTS AND DISCUSSION

Leaf Development Stages Description

The description of the leaf development stages is presented in Figure 1. During Robusta coffee leaf development, the leaf area grows rapidly in the initial days and stabilizes at its maximum size by around 21 days. SPAD increases initially, peaks around 14 days, and then declines slightly. *Pn* follows a similar pattern, increasing to a peak around 14 days and then declining. *Rd* is high initially, decreases as the leaf develops, and stabilizes at a lower level as the leaf matures. The timeline of morphological and physiological events occurring during Robusta coffee leaf development is separated into five stages, modified from Sesták (2012).

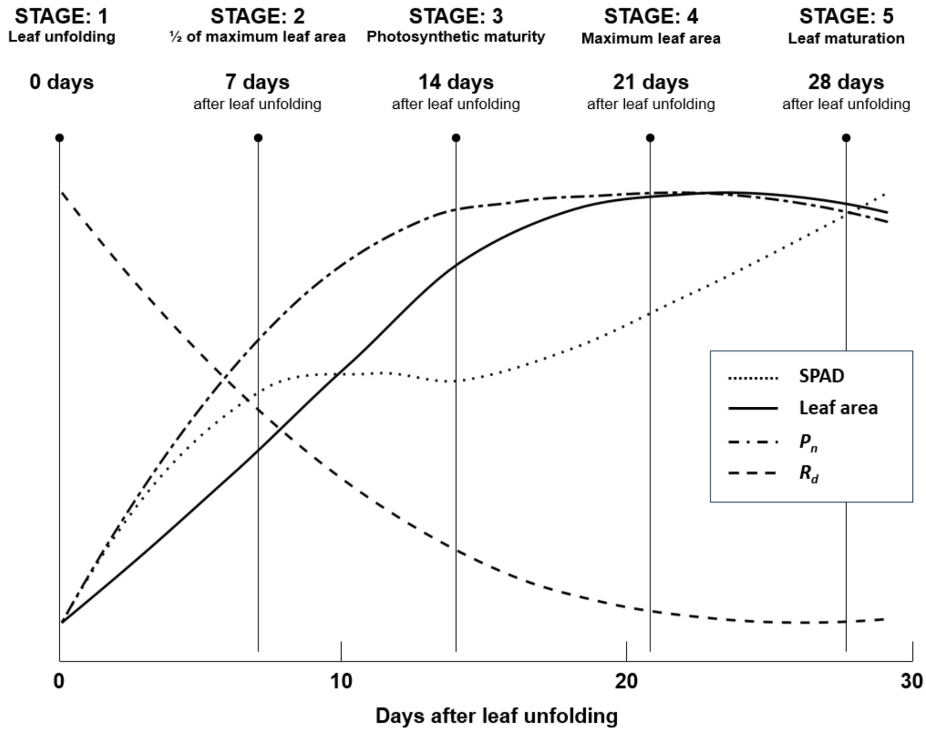

Figure 1 Timeline of average morphological and physiological events occurring during the leaf development of Robusta coffee cv. Chumphon 2 (CH2) and local variety (LO). Stages 1 through 5 represent the progression of leaf development in Robusta coffee: leaf unfolding (stage 1), reaching half of the maximum leaf area (stage 2), attaining photosynthetic maturity (stage 3), achieving maximum leaf area (stage 4), and finally leaf maturation (stage 5) where physiological activities begin to stabilize or decline. Leaf area = single leaf area, SPAD = leaf greenness, *Pn* = net photosynthesis, *Rd* = dark respiration rate (n = 600).

Stage 1: Leaf unfolding (day 0)

During initial leaf development, the paired leaf blades expand to 0.5–1 cm long and unfold after 2–3 weeks. At this stage, CH2 leaves are slender, delicate, and smaller than LO leaves, with CH2 leaves being light green or bronze and LO leaves dark green. Both cultivars form elliptical shapes, and the foliar surface is shiny and waxed (Figure 2A, 2F). At this stage, the following morphological features were observed: the average leaf length for both cultivars was 5.86 ± 0.18 cm with a leaf area of 11.82 ± 0.68 cm^2. The SPAD value was 30.26 ± 1.87. Regarding physiological traits, *Pn* and *Rd* were 0.19 ± 0.11 and 4.04 ± 0.19 μmol CO_2 m^{-2} s^{-1}, respectively. Comparing each cultivar, LO has a higher leaf area and SPAD (Figure 3A-3B), while CH2 shows higher *Pn* and *Rd* (Figure 3C-3D). Low photosynthesis rates during early leaf ontogeny in young rubber trees are attributed to high respiration rates and low chlorophyll content (Schwob *et al*., 1998; Miguel *et al*., 2007). Our results suggest that low chlorophyll content during early leaf development is the primary factor for low photosynthesis rates in coffee.

According to Chen *et al.* (2018), different stages of coffee leaves exhibit variations in phytochemical composition. Young coffee leaves contain more alkaloids, flavonoids, and phenolics than mature leaves. Campa *et al*. (2017) also found that juvenile coffee leaves have higher phenolics, caffeine, and flavonoid concentrations than mature leaves. Chen *et al.* (2018) noted that polyphenol activity decreases during leaf growth. Young leaves contain lower chlorophyll levels due to the photosynthetic apparatus's ongoing development and lower chlorophyll biosynthesis at early stages. This reduced chlorophyll content in young leaves is not related to photoprotection but instead reflects the physiological stage of leaf development (Mullet, 1998).

Stage 2: Leaf expansion to half of the maximum leaf area (day 7)

After the leaf unfolds, the blade expands more in length than width. Both cultivars are delicate and retain a shiny, waxed foliar surface, with CH2 leaves transitioning from bronze to light green (Figure 2B, 2G). Previous studies by Silva *et al*. (2020) on young *Eucalyptus* leaf ontogeny observed similar color changes in young *Eucalyptus* leaves, in which reddish tones gradually turned green during leaf expansion stages B1, in which reddish tones are predominant, and B2, in which green tones become more pronounced.

During leaf development from stage 1 to stage 2, the leaf area expands to half its maximum size (46.39 ± 12.15 cm^2 at stage 2, from a maximum leaf area of 99.82 ± 21.74 cm^2). All traits increased linearly from stage 1 to stage 2 (Figure 1). The greatest slope values from linear regression between each parameter were observed in leaf area (4.48), SPAD value (1.39), *Pn* (0.35), and *Rd* (0.19). At this stage, SPAD value, *Pn*, and *Rd* show the highest growth rates compared to other stages (Table 1). Comparing the cultivars, LO still has higher SPAD values, CH2 still has higher *Rd* (Figure 3B, 3D), and leaf area and *Pn* had switched (Figure 3A, 3C).

At stage 2, the low photosynthesis rate suggests that young leaves are a typical sink during early development, which limits their ability to supply assimilates to other sink tissues. During leaf expansion, as indicated by high slope values of leaf area, *Pn*, and *Rd*, cell division transitions to cell enlargement when leaves reach about one-third to half of their maximum area (Sesták, 2012). Initially, the leaf imports carbon and remains a net importer until its photosynthetic capacity fully develops and the demand for photosynthates decreases.

Stage 3: Photosynthetic maturity of the leaf (day 14)

Both cultivars still have delicate, shiny, waxed foliar surfaces (Figure 2C, 2H). The photosynthetic parameters (*P*n) show a period of 'photosynthetic maturity', with slopes tending towards zero. During leaf development from stage 2 to this stage, the leaf area shows the most significant increase, with a growth rate slope of 6.42 (Table 1). Both *P*n and *R*d also increased (0.13 and 0.15, respectively). In contrast, the SPAD value decreased (-0.10) compared to the previous stage (Table 1).

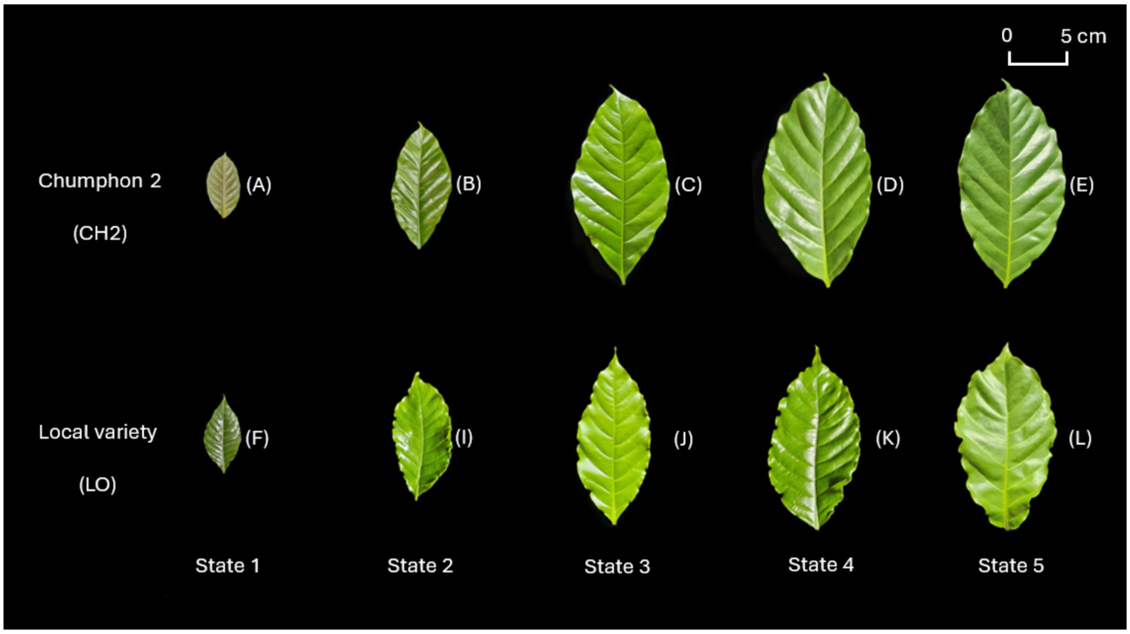

Figure 2 Leaves from five leaf-age stages in Robusta coffee cultivars Chumphon 2 (CH2) and local variety (LO) are shown. State 1 represents 0 days after the leaf pair unfolding, state 2 represents 7 days, state 3 represents 14 days, state 4 represents 21 days, and state 5 represents 28 days. These stages are denoted by A-E for CH2 and F-L for LO, respectively.

Table 1 The slope value of the linear regression model represents the relationship between changes in morphological and physiological parameters and the days of Robusta coffee leaf development at each growth stage

Parameters	Stages 1 to 2		Stages 2 to 3		Stages 3 to 4		Stages 4 to 5	
	Slope	R^2	Slope	R^2	Slope	R^2	Slope	R^2
Leaf area	4.48	0.99	6.42	1.00	2.00	0.91	0.00	0.50
SPAD	1.39	0.95	-0.10	0.13	0.17	0.37	0.35	0.96
Pn	0.35	0.89	0.13	0.93	-0.04	0.19	0.00	0.07
Rd	-0.19	0.84	-0.15	0.79	-0.08	0.89	0.00	0.01

Note: Leaf area = single leaf area, SPAD = leaf greenness, *Pn* = net photosynthesis, *Rd* = dark respiration rate (n = 140). State 1 represents 0 days after the leaf pair unfolding, state 2 represents 7 days, state 3 represents 14 days, state 4 represents 21 days, and state 5 represents 28 days.

The increase in *Pn* could be due to increasing chloroplast maturation; as the leaf matures, chloroplasts become more fully developed and efficient in capturing light and converting it into chemical energy. The increase in photosynthetic activity (*Pn*) could be attributed to the improved functionality of these chloroplasts (Sesták, 2012). The decrease in SPAD value may be explained by the increase in plastids in palisade cells being highest before the period of maximum leaf expansion. By contrast, the most significant increase in the total number of plastids occurs during the most remarkable leaf expansion period, when cell division has stopped, and the plastids have all developed into chloroplasts (Whatley, 1980). The change in chloroplast density was caused by the growth of leaf cells, which led to a decrease in the number of cells per leaf surface unit. As a result, the chloroplasts in the leaves spread out more. This spreading was further influenced by the differentiation and expansion of palisade and spongy parenchyma cells (Sesták, 2012), during leaf development under shaded conditions, chloroplasts often redistribute within the cell to optimize light absorption. Instead of being evenly distributed, they tend to align along the cell walls to maximize their exposure to available light. This strategic positioning allows the chloroplasts to capture as much light as possible, which is crucial for efficient photosynthesis under low-light conditions (Wada *et al*., 2003).

Stage 4: Leaf expands to maximum leaf area (day 21)

At this stage, the leaf blade becomes fully expanded. From stage 3 to this stage, the leaf area, SPAD value, and *R*d increase slightly (slopes of 2.00, 0.17, and 0.08, respectively), while *P*n decreases slightly (slope of -0.04; Table 1). The leaf area is higher in CH2 than in LO. Leaf area expansion is crucial for enhancing light harvesting and varies among plant types. Studies on species such as sunflower (de Carvalho, 2004) and sugarcane (Almeida *et al.*, 2008) indicate that leaf area expansion depends on changes in sucrose metabolism during the sink-to-source transition.

Stage 5: Leaf maturation (day 28)

When the leaves reached this last stage, all the morphological (leaf area) and physiological (*P*n and *R*d) parameters remained unchanged, except the SPAD value, which still increased in slope to 0.35. In this stage, leaf maturity is marked by full development in size and function, with a transition from high metabolic activity to maintenance. This transition includes a decrease in dark respiration rates as the energy requirements for growth diminish and the leaf's role shifts to sustaining photosynthesis and supporting other parts of the plant (Bielczynski *et al*., 2017).

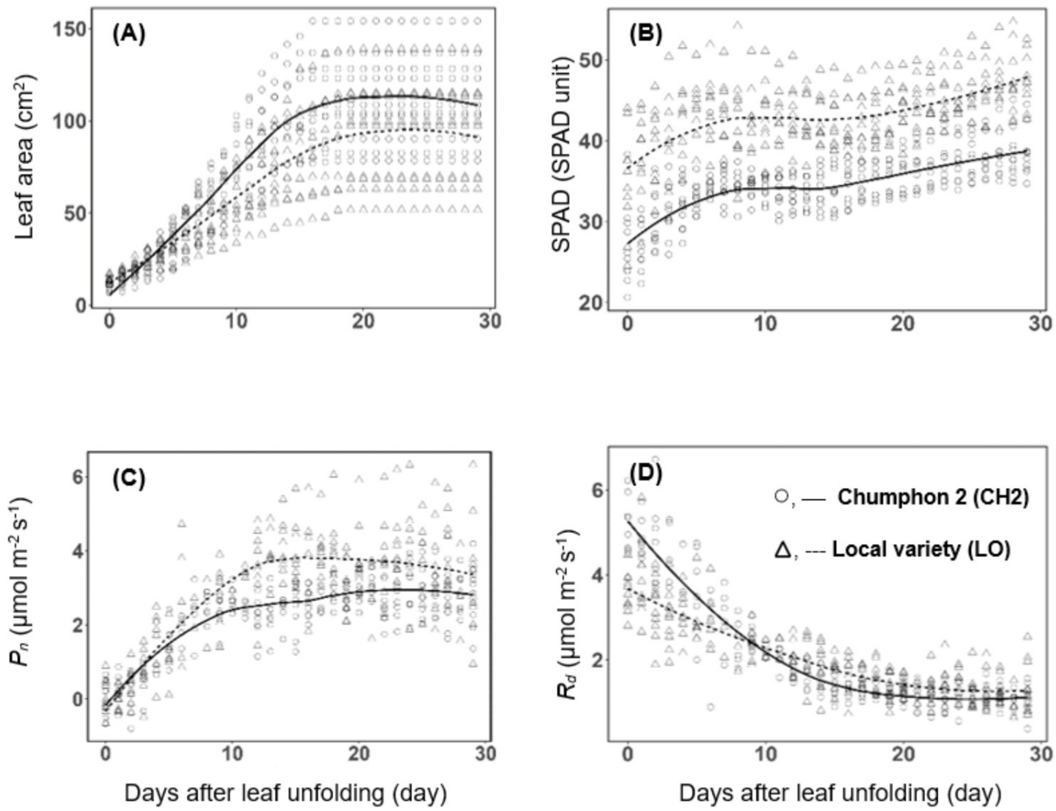

Figure 3 Local polynomial regression fitting curves of single leaf area (A), leaf greenness (SPAD; B), net photosynthesis (*Pn*; C), and dark respiration rate (*Rd*; D) according to Robusta coffee cultivars (N = 300). (○, —) indicates Chumphon 2 (CH2), (▲, ---) indicates local variety (LO).

Correlation between Morpho-physiological Traits in Robusta Coffee

The correlation matrix illustrates the Pearson correlation coefficients between SPAD, leaf area, *Pn*, and *Rd* for the Robusta coffee cultivars CH2 and LO. The analysis shows that compared to LO, CH2 had stronger positive correlations between SPAD and both leaf area (r = 0.590; P < 0.001) and *Pn* (r = 0.668; P < 0.001), and a stronger negative correlation exists between SPAD and *Rd* (r = -0.622; P < 0.001). Similarly, the leaf area in CH2 demonstrates a more robust positive correlation with *Pn* (r = 0.624; P < 0.001) and a stronger negative correlation with *Rd* (r = -0.834; P < 0.001). Additionally, *Pn* exhibited a more pronounced negative correlation with *Rd* in CH2 (r = -0.759; P < 0.001) than in LO. These findings indicate that the physiological interactions between SPAD, *Pn*, and *Rd* are more tightly linked in CH2 than LO (Figure 4).

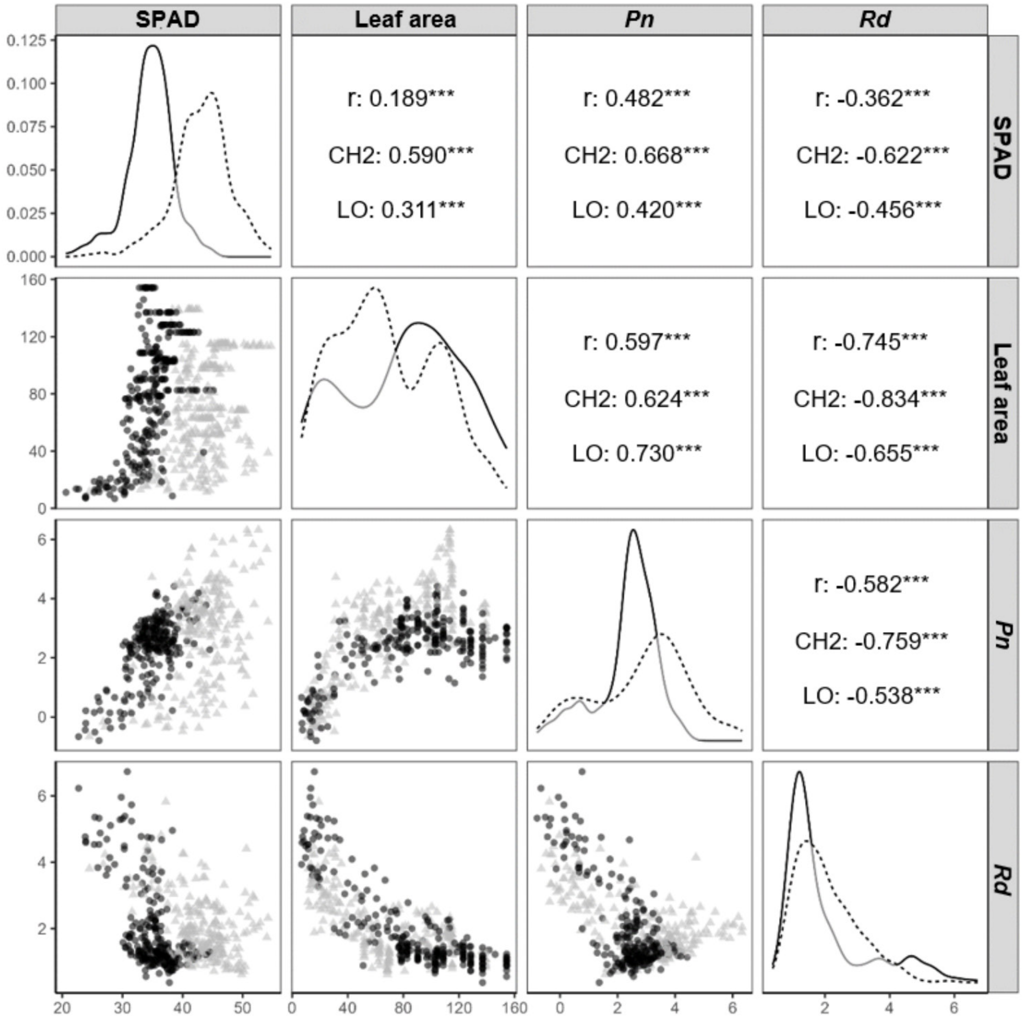

Figure 4 Pearson's correlation between leaf greenness (SPAD), single leaf area, net photosynthesis (*Pn*), and dark respiration rate (*Rd*) of Robusta coffee cultivars (N = 300). (●, ──) indicates Chumphon 2 (CH2) and (▲, ---) indicates local variety (LO). The asterisk represents a significant correlation. * 0.01 < P < 0.05; ** 0.001 < P < 0.01; *** P < 0.001.

The two varieties, CH2 and LO, exhibit different correlations among the measured variables, reflecting their adaptability and potential performance under shaded conditions. LO has a more substantial potential for growing in shaded environments. It shows a stronger correlation between leaf area and net photosynthesis, indicating that it can better utilize its leaf area for energy production even with limited light. The less negative correlation between dark respiration rate and the other variables suggests that LO is more energy-efficient and crucial in shaded environments where light is scarce. This study lays the foundation for further exploration and the selection of coffee varieties that are not only productive but also environmentally sustainable.

CONCLUSIONS

The total leaf development duration for young Robusta coffee cultivars CH2 and LO under 80% shade and without environmental stress ranges from 28–30 days. Five stages represent significant changes in leaf development, including size, photosynthesis, and dark respiration. Stage 1 (0 days): leaf pair unfolding begins. CH2 leaves may appear bronze, while LO leaves are green. Stage 2 (7 days): leaf blades expand to half their maximum area, with CH2 leaves transitioning from bronze to green. Stage 3 (14 days): photosynthetic capacity peaks, and leaf greenness stabilizes. Stage 4 (21 days): during this stage, leaves reach full size, and greenness increases again. CH2 leaves become longer, while LO leaves become broader. Stage 5 (28 days): dark respiration stabilizes, leaf greenness increases, and leaves become thicker and stronger. Physiological traits increase throughout development. Early stages show the stability of the coefficient of determination or R^2 values. However, from stages 2 to 3, R^2 increases, indicating a critical sensitive period for leaf size changes between 7 to 14 days after unfolding.

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