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How Cropping System Influences Robusta Coffee Leaf Gas Exchange Efficiency *via* Stomatal Regulation

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ABSTRACT

Background: Robusta coffee leaves regulate gas exchange in response to climatic factors like full sun and shaded conditions. To fully understand the reasons behind the variations in photosynthetic rates and water-use efficiency, exploring the underlying mechanisms of stomatal regulation in the maximum photosynthetic efficiency of Robusta coffee trees, especially in a shade condition is necessary.

Methods: This study aimed to evaluate how stomatal regulation in Robusta coffee leaves affects gas exchange under different cropping systems. The Robusta coffee trees were grown in four different cropping systems: monoculture (full-sun condition) (S1), intercropped (single row) with rubber trees (S2), intercropped (double spacing) with rubber trees (S3), and intercropped in a mixed orchard (S4).

Result: The results revealed that Robusta coffee trees grown between rubber tree rows with closer spacing (S2) exhibited the largest stomata sizes. These stomata sizes had a statistically significant increase in width, length, and area (ranging from 12.04 to 14.74 µm, 19.22 to 25.55 µm and 202.37 to 296.05 µm^2, respectively) compared to Robusta coffee grown in S1, S3 and S4 systems. On the other hand, Robusta coffee grown in S1 system displayed the highest stomatal density, ranging from 310 to 540 stomata/mm^2. Interestingly, coffee trees grown between rubber rows (S3) and in the mixed orchard (S4) exhibited both high rates of photosynthesis (9.18 and 9.35 µmol/m^2/s) and photosynthetic water-use efficiency (3.78 and 5.15 mmol/mol). Notably, these values were statistically different from those observed in other planting conditions. Additionally, Robusta coffee grown in S1 system exhibited the highest canopy temperature (ranging from 30.8 to 47.4°C). It also had the lowest F_v / F_m value (ranging from 0.706 to 0.759) compared to other planting conditions. This study indicates that Robusta coffee leaves exhibited varying stomatal responses to changes in light and temperature depending on the cropping system. By understanding how these leaf traits influence adaptation, we could potentially optimize Robusta coffee trees to thrive in full sun and shaded conditions, particularly within intercropping systems.

Key words: Intercropping system, Leaf traits, Phenotypic adaptation, Shade tolerance, Water-use efficiency.

INTRODUCTION

Stomata are microscopic pores essential for regulating gas exchange on the leaf surface. They control the movement of gases in and out of the leaf, facilitating the influx of carbon dioxide and the efflux of oxygen (Willmer and Fricker, 1996). The distribution of stomata varies greatly across different plant species. Interestingly, light intensity and carbon dioxide concentration have been shown to influence the density of stomata on leaves (Casson and Gray, 2008). Stomata control gas exchange and significantly impact plant health and function by regulating both transpiration and photosynthetic rates (Hong *et al*., 2018). Stomatal features, including their evolution to balance efficient CO_2 uptake with water regulation, allow plants to adapt to varying atmospheric CO_2 levels (Hu *et al*., 2019). Balancing photosynthesis with water conservation is a complex task for plants, requiring intricate control of stomata. Evolutionary trade-offs determine the relationship between stomatal size and density, which can be further affected by CO_2 levels, potentially impacting crop productivity (Haworth *et al*., 2022). Regulators of gas exchange not only determine how much CO_2 plants capture for growth but also influence water loss (Haworth *et al*., 2021).

As climates change, water use efficiency becomes increasingly critical for coffee plants. Stomata, tiny pores on leaves, act as control points, balancing the essential uptake of CO_2 for photosynthesis with the inevitable loss of water vapor (Tounekti *et al*., 2018). By regulating stomatal development and traits, plants can improve their water-use

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efficiency and become more drought tolerant (Bertolino *et al*., 2019). Morphological and stomatal differences were also significantly pronounced between susceptible and tolerant cultivars under high-temperature stress conditions (Sabina and Sameena, 2022). Coffee plants, like Robusta coffee, exhibit paracytic stomata, a type where guard cells flank the stomata parallel to the pore (Nattawit *et al*., 2019). In Magnoliaceae species, stomata size can be estimated using their length and width. These tiny pores also respond to environmental changes and adjust their shape and size to regulate water balance in the plant (Zhang *et al*., 2023). Meanwhile, blueberry cultivars have varying optimal growth temperatures, with excessive heat negatively affecting their growth, physiology and even leaf and chloroplast structure (Zheng *et al*., 2017). Olive trees suffer negative impacts on photosynthesis and stomatal function when exposed to heat stress. This can be detected through chlorophyll fluorescence analysis, which provides insights into plant stress responses (Haworth *et al*., 2018). The crop's good adaptation to drought stress was associated with maximum chlorophyll content, higher relative water content, and increased stomatal number, leading to greater dry matter accumulation (Patel *et al*., 2022). Interestingly, the distribution of stomata might not be evenly distributed but rather exhibit strong spatial clustering of stomata across the leaves (Martins *et al*., 2012).

Since light intensity is lower in shaded conditions, Robusta coffee plants have adapted leaf shapes and mechanisms to enhance their light capture for photosynthesis by expanding leaf area and chlorophyll and carotenoid contents (Nattawit *et al*., 2019). Coffee plants require light for photosynthesis and open their stomata to absorb CO_2. However, they face the constant challenge of balancing CO_2 uptake with water loss through the stomata pores (Pompelli *et al*., 2010). In agroecosystems where coffee is intercropped with rubber trees, Robusta coffee experiences additional limitations due to competition for light and root space (Chiarawipa *et al*., 2021).

Therefore, this study investigated the effect of light intensity and temperature on the stomata of Robusta coffee leaves in different cropping systems. This study could improve the light management practices in rubber-coffee intercropping and mixed orchard systems.

MATERIALS AND METHODS

Experimental site and planting

The experiment was conducted from November 2022 to May 2023 on Robusta coffee trees that were intercropped in rubber farms and mixed orchard in Songkhla province (latitude 6°44′56.0"N, longitude 100°40′24.0"E, and altitude 35 m above sea level), southern Thailand. The Robusta coffee trees were grown in four different cropping systems: 1) monoculture (full-sun condition) planted in a spaced of 3×7 m (S1), 2) intercropped (single row) with rubber trees in a spacing of 3×7 m (S2), 3) intercropped with (3 rows) with rubber trees in a double spacing of 3×13 m (S3) and 4) intercropped in a spaced of 3×3 m in a mixed orchard comprised of banana, lime, and salak (*Salacca zalacca*) (S4). All rubber trees in S2 and S3 were RRIM 600, which were 15 and 5 years old, respectively and represented the high and low-storey conditions of the farms. All Robusta coffee trees in this study were five years old and sample leaves were collected from each plot.

Climate variables

Weather conditions were monitored using a micro weather station that continuously recorded climate variables for each plot. The air temperature (°C) and relative humidity (%RH) were recorded at hourly intervals using a data logger to determine monthly means (DT-172, China). To evaluate the monthly light intensity, photosynthetically active radiation (PAR) was measured every week between 11:00 to 13:00 hr in the inter-row using a Light meter (Sun System, Canada).

Gas exchange measurements and chlorophyll fluorescence measurements

Leaf gas exchanges were measured in the youngest fully expanded leaves of five individual Robusta coffee plants per treatment using a Portable Photosynthesis System (LI-6800, LI-COR, Lincoln, NE, USA). The system was equipped with a controlled light source of 1,200 µmol/m^2/s and set to the following conditions: CO_2 concentration of 400 ppm, relative humidity of 70±5% and leaf temperature of 30±2°C. Then, the value of photosynthetic water-use efficiency (PWUE) was estimated in each treatment in which coffee leaves utilize water for CO_2 fixation during photosynthesis. It was calculated as the ratio of the net assimilation rate (A) (µmol/m^2/s) to the transpiration rate (E) (mmol/m^2/s). In addition, the rate of stomatal conductance (g_s) (mmol/m^2/s) was recorded.

A portable chlorophyll fluorescence meter (Handy PEA+, Hansatech Instruments, UK) was used to measure the maximum photochemical efficiency (F_v/F_m) of photosystem II in Robusta coffee leaves. This involved determining the ratio of variable fluorescence (F_v) to maximum fluorescence (F_m) in dark-adapted leaves in 30 min. Leaf greenness was measured using a chlorophyll meter (Dualex, DX19007, Force A, France) and 2 to 4 leaf pairs were randomly selected from the shoot tips. The change in temperature of Robusta coffee canopy was measured using a thermal infrared camera (Thermal Infrared Camera, Testo 875-2i, Germany). All physiological responses were measured in the midday (11:00 to 13:00 hr).

Leaf stomatal traits measurements

Stomatal traits of Robusta coffee plants were quantified by a rapid stomatal phenotyping method using the nail polish method described by Pathoumthong *et al*. (2023). This method provides information about the density and distribution of stomata on the Robusta coffee leaf surface. Briefly, a thin layer of nail polish was applied to the upper (adaxial) surface of each leaf. After drying, the polish was carefully peeled off with clear tape, creating a replica of the

stomata. Stomatal width, length and density were analyzed from temporary slides containing five coffee leaf samples per treatment. Each leaf was divided into three sections: base, middle and apex. Five replicates were analyzed from each section at 40x magnification using an inverted microscope (IX73, Olympus, Tokyo, Japan) and the CellSens Standard version 3.1 image analysis software at Plant Ecophysiology Laboratory (PSU_NATRES13) situated in the Faculty of Natural Resources, Prince of Songkla University, Songkhla, Thailand.

To compare the leaf shape and size across the different cropping systems, stomatal sizes were estimated using the Montgomery equation (Li *et al*., 2022). This model defines that stomatal area is directly proportional to the product of its length and width. The planar area is enclosed by the two guard cells. The longest dimension between the guard cell edges was taken perpendicular to the stomatal opening. Then, the maximum distance between the lines formed by the stomatal edges was measured perpendicular to the longer axis of the stoma. The Montgomery equation, which utilizes stomatal length (L) and width (W), was employed to estimate the area of individual stomata (A). This involved analyzing the relationship between the natural logarithm of stomatal area and the product of the natural log-transformed length and width as described by Zhang *et al*. (2023).

$$[\ln(A) = a + \ln(LW)]$$

Linear regression was then employed to estimate the model parameters. Therefore, the model based on stomatal length and width was used to predict the area of individual stomata.

Statistical analysis

Data of physiological responses, leaf greenness, and stomatal traits were compared among the treatments using one-way analysis of variance (ANOVA) with a completely randomized design. Least significant difference (LSD) was performed to identify significant differences ($p<0.05$) between the treatments, using R software.

Results and Discussion

Weather conditions

Among the experimental plots, the full-sun Robusta coffee trees planted in S1 system had the highest light intensity, reaching 1,761.85 µmol/m^2/s. Conversely, the S2 plot where Robusta coffee trees planted in a single row in the rubber trees' interrow had the lowest light intensity, at 727.13 µmol/m^2/s. Meanwhile, the S3 and S4 systems had similar light intensity, measuring 1,534.52 and 1,594.90 µmol/m^2/s, respectively. Interestingly, all four plots exhibited similar air temperatures and relative humidity, ranging from 26.50 to 30.32°C and 71.27 to 88.79% (Fig 1).

Stomatal sizes and density of Robusta coffee leaves

The stomata of Robusta coffee trees grown in S2 system had a maximum size of 12.04 to 14.74 and 19.22 to 25.55 µm. These sizes were statistically different from those of Robusta coffee trees grown in S1, S4, and S3 systems. The size of the stomata had widths of 11.62 to 13.96, 10.88 to 13.74, and 10.37 to 13.15 µm, respectively, and lengths of 16.49 to 24.21, 17.60 to 23.73 and 17.32 to 19.96 µm, respectively. However, the ratio of width to length of Robusta coffee leaf stomata was not statistically different (Fig 2).

Robusta coffee trees grown as a monoculture (S1) exhibited the highest stomatal density, ranging from 310 to 540 stomata/mm^2. This value was not statistically different from that of Robusta coffee trees grown with rubber trees planted in S2 (200 to 400 stomata/mm^2). However, it was statistically different from that of Robusta coffee trees in S3 system (250 to 460 stomata/mm^2) and S4 system (210 to 380 stomata/mm^2), which had the lowest stomatal density

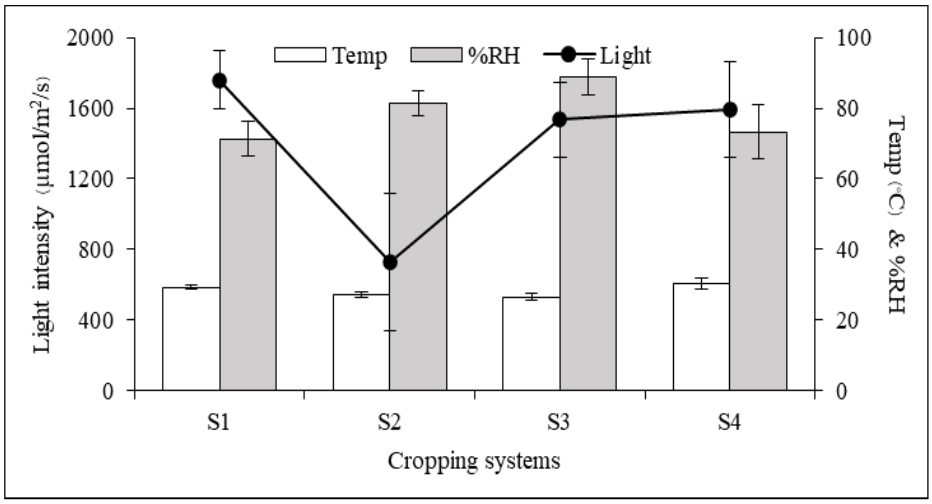


Fig 1: Air temperature (Temp), relative humidity (%RH) and light intensity (Light) in the experimental sites.

(Fig 3). The stomata of Robusta coffee trees in S2 system exhibited the largest area, ranging from 202.37 to 296.05 µm^2 and were statistically different from the other treatments. Robusta coffee trees in S1 and S4 systems had similar stomatal areas, ranging from 151.06 to 258.99 µm^2 and 163.02 to 240.99 µm^2, respectively. Finally, Robusta coffee trees in S3 system had the lowest stomatal area (156.76 to 197.82 µm^2) and were statistically different from the other treatments. An evaluation of the relationship between stomatal area (A) and leaf width (LW) revealed a linear relationship. This could be expressed by the equation y= 1.2727x+0.0001, with a strong correlation coefficient (r^2= 0.999). Interestingly, a logarithmic transformation of both variables [Ln(A) and Ln(LW)] also showed a strong linear relationship (y= 1x - 0.2412, r^2= 0.999) (Fig 4).

Robusta coffee trees in S2 system exhibited the largest stomatal size compared to other planting systems. This finding suggests a potential adaptation to shaded conditions. Larger stomatal size could facilitate increased gas exchange, which might benefit photosynthesis in lower-light environments. Plants possess a remarkable ability to adapt to their environment by adjusting the size and number of stomata on their leaves, responding to various environmental cues (Harrison *et al*., 2020; Baby *et al*., 2023). Then, this study shows that Robusta coffee leaves in S1, S3 and S4 systems responded to higher light intensity by regulating water loss through stomatal closure. They achieved this by closing their stomata in width, length and overall area. Moreover, light intensity did not influence the fundamental shape of the coffee leaf stomata (paracytic or Rubiaceous type). However, leaf development significantly affects stomatal size (width and length) and density (Willmer and Fricker, 1996). In this case, reduced light availability due to the presence of rubber trees (3×7 m) (S2) might have triggered an increase in stomata size to compensate for lower light levels, while stomata density might have been adjusted to regulate water loss. In contrast, the Robusta coffee trees grown with rubber tree (3×13 m) (S3) and mixed orchard (S4), which received higher light intensity compared to the closer planting distance (3×7 m)

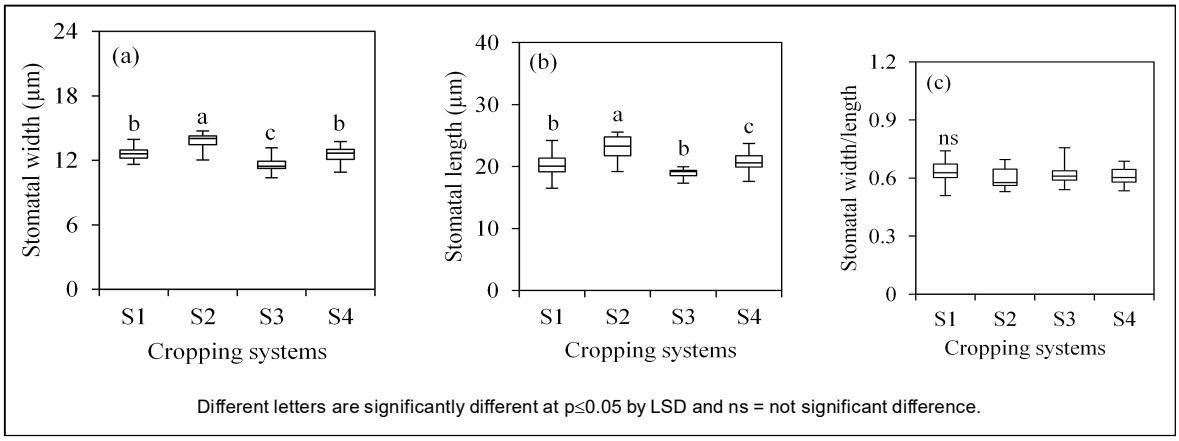


Fig 2: Box plots of stomata width (a), stomata length (b) and stomata width/length (c) in Robusta coffee leaves.

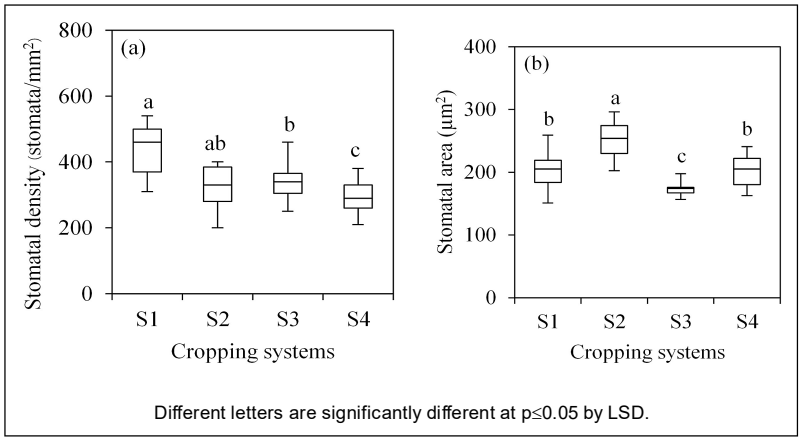


Fig 3: Box plots of stomatal density (a) and stomata area (b) in Robusta coffee leaves.

(S2), exhibited smaller stomata and lower stomatal density. This is likely an adaptation to the higher light environment. Smaller stomata with reduced depth can facilitate faster diffusion of CO_2 into the leaf interior (Franks and Farquhar, 2007). This, in turn, could enhance the availability of CO_2 for photosynthesis within the leaf intercellular spaces (Drake *et al*., 2013). Additionally, lower stomatal density under high light conditions is a well-established strategy for coffee plants to balance water use and CO_2 uptake. This adaptation could improve drought tolerance and reduce water loss (Harrison *et al*., 2020).

Similarly, tropical palm species grown under the light transmitted through the rubber tree canopy exhibited significant adaptive stomatal dynamics. This adaptation involved a decrease in guard cell size (both width and length), leading to a reduction in stomatal pore size.

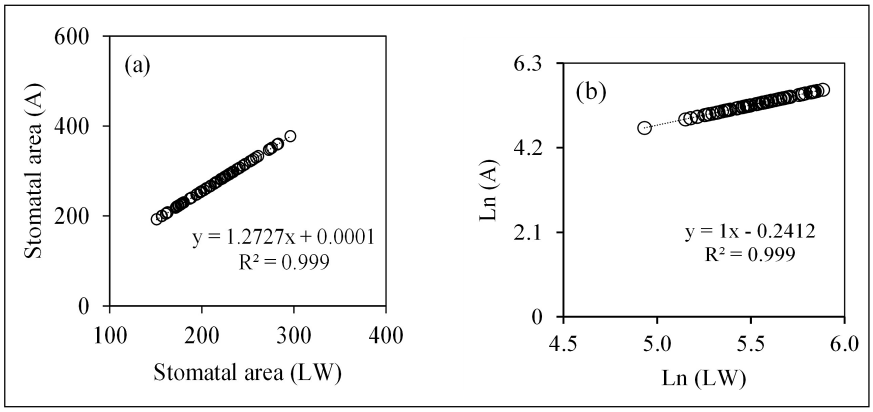


Fig 4: Relationships between stomatal area (A) and the product of stomatal length (L) and width (W) (a) and relationships between stomatal area (A) and the product of stomatal length (L) and width (W) on a log-log scale (b) for Robusta coffee leaves.

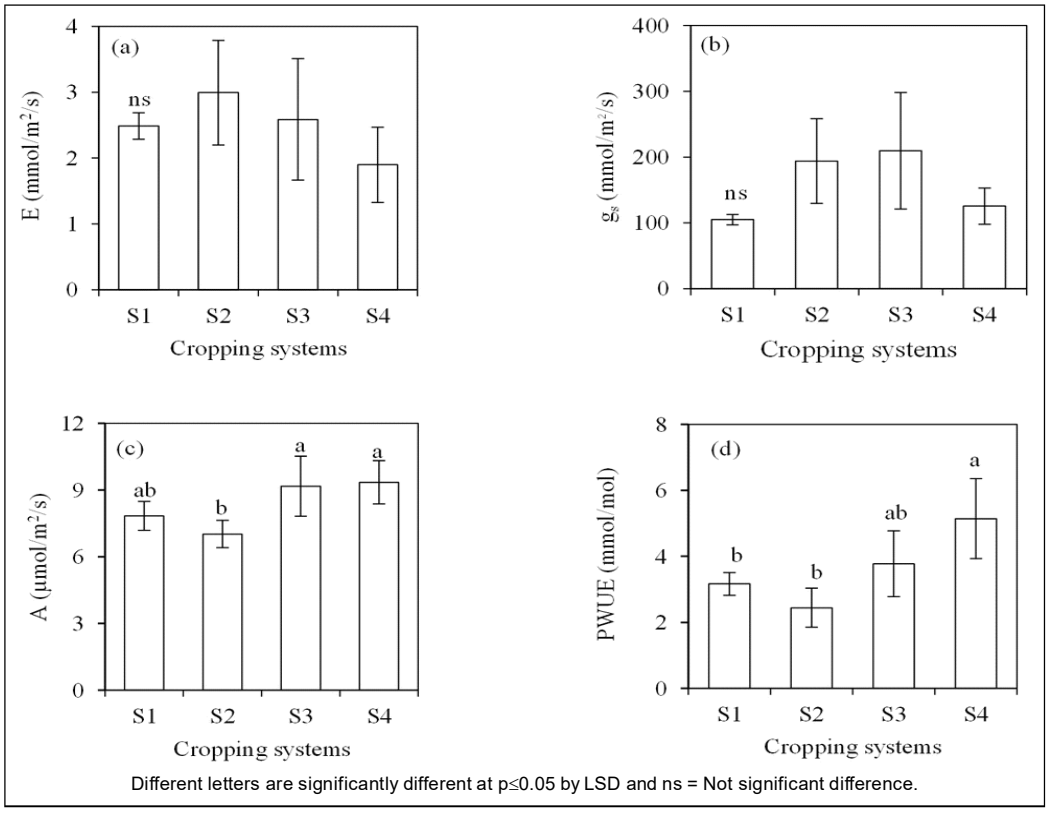


Fig 5: Effect of cropping system on transpiration rate (a), stomatal conductance (b), photosynthetic rate (c) and photosynthetic water-use efficiency (d) in Robusta coffee leaves.

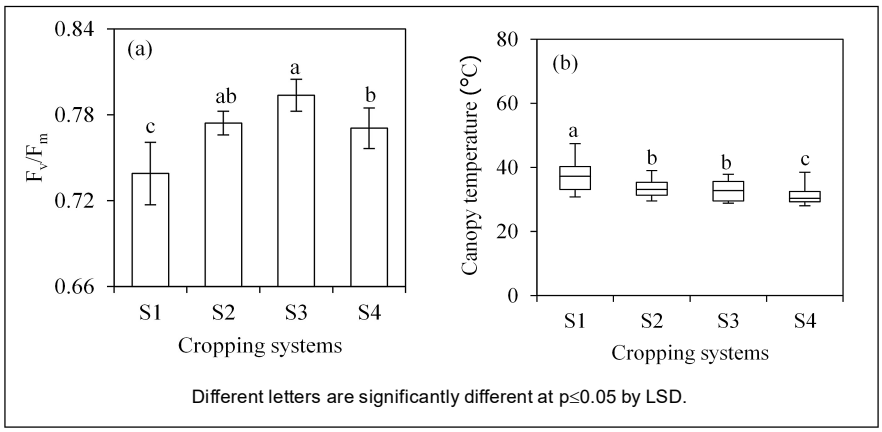


Fig 6: Effect of cropping system on the maximum quantum efficiency of photosystem II (F_v / F_m) (a) and canopy temperature (b) in Robusta coffee leaves.

The smaller pore size likely served to minimize leaf transpiration rates under the conditions of lower light availability (Zaw *et al*., 2023). This result shows that Robusta coffee is a tropical fruit tree species that primarily adjusts its stomata in response to changes in light intensity, not water availability like other tropical plant species (Bertolino *et al*., 2019).

Physiological responses of Robusta coffee leaves

Robusta coffee trees in S3 and S4 systems exhibited high rates of photosynthesis (9.18 and 9.35 µmol/m^2/s) and photosynthetic water-use efficiency (PWUE) (3.78 and 5.15 mmol/mol). These values were statistically different from those observed in other planting conditions. Notably, the photosynthetic rates were similar between the S3 and S4 systems. In contrast, Robusta coffee trees in S2 system had a lower photosynthetic rate (7.03 µmol/m^2/s) that was not statistically different from S1 system. Furthermore, the photosynthetic water-use efficiency of Robusta coffee trees grown in S1 system (3.17 mmol/mol) and S2 system (2.44 mmol/mol) was similarly low. Interestingly, no statistically significant differences were observed in transpiration and stomatal conductance rates across all four planting systems (Fig 5).

Among the Robusta coffee trees, those trees planted with rubber trees in S3 system exhibited the highest chlorophyll fluorescence (F_v / F_m) value, at 0.794. In contrast, Robusta coffee trees in S2 and S4 systems had similar values (0.774 and 0.771). Robusta coffee trees grown at S1 system had the lowest F_v / F_m value, at 0.739. Moreover, S1 system had the highest canopy temperature, ranging from 30.8 to 47.4°C. In contrast, Robusta coffee trees in other planting systems had similar temperatures: 29.6 to 39.0°C for S2 system, 28.8 to 37.9°C for S3 system and 28.0 to 38.5°C for S4 system, respectively (Fig 6).

The physiological responses reveal that Robusta coffee trees grown with rubber trees in S3 and S4 systems exhibited higher photosynthetic rates and water-use efficiency compared to S1 and S2 systems. This suggests that Robusta coffee trees have a better adaptation to these light conditions. Robusta coffee trees demonstrated remarkable adaptability, thriving in various light intensities, from full sun to environments with some shade. While Arabica coffee benefits from moderate shade levels (up to 55%) for maintaining photosynthesis compared to full sun (Franck and Vaast, 2009), Robusta coffee also thrives under even greater shade (41-65%). This is likely due to Robusta coffee's ability to manage stomatal stress. By reducing stomatal stress significantly, Robusta coffee tree could continue effective photosynthesis at lower light levels, leading to higher productivity and growth under shade conditions (Piato *et al*., 2020). Also, Robusta coffee tree could be sensitive to both insufficient and excessive light intensity, potentially leading to reduced physiological performance such as photosynthetic and transpiration rates in coffee grown under shade (Morais *et al*., 2004). Interestingly, there was no statistical difference in stomatal conductance or transpiration rate detected. However, a trend of lower stomatal conductance was observed, which could potentially explain the decrease in transpiration.

In response to high light intensity, Robusta coffee trees grown intercropped with rubber trees at a closing space might experience limitations due to light availability. Robusta coffee leaves tended to be smaller and developed more serrated or overlapping edges. This adaptation helps to dissipate heat energy from sunlight more efficiently to protect against the sunburn disorder of the leaf blade. In contrast, leaves in low-light environments tend to be larger and smoother-edged to maximize their light-absorbing surface area (Nattawit *et al*., 2019). Moreover, lower F_v / F_m values, in the range of 0.706 to 0.759 observed in the full-sun condition, suggest that the Robusta coffee canopy might be experiencing heat stress (Kulasin *et al*., 2022), potentially leading to inhibited photosynthesis more than in other cropping conditions (Tounekti *et al*., 2018). To fully understand the reasons behind the observed variations in photosynthetic rates and water-use efficiency, further research is necessary to explore the underlying mechanisms of stomatal

regulation in the maximum photosynthetic efficiency of Robusta coffee trees.

Plant success depends on the adaptability of stomata, which are influenced by both genetic variation and environmental factors (Sabina and Sameena, 2022; Baby *et al.*, 2023). This study shows that environmental changes within an agricultural ecosystem could influence the development of stomata in Robusta coffee trees by affecting the pathways that control their number and arrangement. Then, light intensity is a major environmental factor affecting stomatal density in the cropping system. Robusta coffee trees grown in the shade of rubber trees and fruit trees have lower stomatal density compared to those grown in full-sun conditions. This reduction in stomata likely helps conserve water in shaded environments, as stomata regulate gas exchanges. Understanding these stomatal characteristics is crucial for optimizing light management in intercropping Robusta coffee with rubber trees and mixed orchard systems to enhance the understory's acclimatization to shade.

CONCLUSION

Manipulating stomatal traits like density and morphology presents a promising approach to enhancing shade tolerance in Robusta coffee trees. Planting Robusta coffee with rubber trees at a closer spacing (3×7 m) led to the largest stomata size (length, width, and area) and stomata density. However, under slightly shaded conditions, such as those found in rubber plantations with a double row spacing (3×13 m) and the mixed orchard system, Robusta coffee leaves exhibited a better physiological response, particularly in terms of photosynthesis and water-use efficiency.

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Conflict of interest

All authors declared that there is no conflict of interest.

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