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ORIGINAL ARTICLE

Spatiotemporal variation in *Coffea canephora* leaf traits and iWUE in Congo Basin forests

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- **Background and Aims** Understanding spatiotemporal variation in plant functional traits and intrinsic water use efficiency (iWUE) is essential to evaluate how plants respond to environmental change. In forests of the Congo Basin, we examined spatial and century-scale temporal trends in the morphological and physiological characteristics of the leaves of *Coffea canephora*, a widespread understorey species from West Africa to the African rift (Uganda).
- **Methods** Using 179 herbarium samples collected during two periods (1900–60 and 2016–21), we measured the specific leaf area (SLA), stomatal size (S), stomatal pore size (SPS), stomatal density (SD) and maximum diffusive stomatal conductance to CO_2 (g_{cmax}). Stable carbon and oxygen isotope ratios ($\delta^{13}C$, $\delta^{18}O$) were measured from leaf cellulose to infer variation in photosynthetic activity iWUE.
- **Key Results** We found a significant spatiotemporal variation in leaf morphological and physiological traits and iWUE. $\delta^{13}C$ ranged from −34.84 to −24.11 ‰, and $\delta^{18}O$ from +26.96 to +34.16 ‰. Over the past century, SLA and S increased, whereas SPS, SD, g_{cmax}, $\delta^{13}C$ and iWUE decreased. Spatially, morphological traits appeared shaped by long-term environmental adaptation, while physiological traits responded more to short-term drivers such as atmospheric CO_2 and precipitation, highlighting a functional decoupling that may limit photosynthetic performance of *C. canephora* under future climate change. The trait correlations showed coordinated functional trade-offs: SLA was negatively correlated with iWUE, while S, SD and g_{cmax} were positively associated, reflecting trade-offs between carbon gain and water conservation.
- **Conclusions** Our study underscores the value of herbarium-based multitrait approaches in reconstructing long-term plant responses and their relevance for understanding climate sensitivity in tropical understorey species.

Key words: Carbon-13, coffee, global change, oxygen-18, stomatal conductance, tropical forests.

INTRODUCTION

Human activities continue to lead to increased mean annual global greenhouse gas (GHG) emissions, making climate change a global threat, affecting multiple sectors of global development (IPCC, 2023). Among the most relevant manifestations of global climate change are the increase in global mean surface temperature (Rahman *et al.*, 2019; IPCC, 2023), altered rainfall patterns and an increase in extreme weather events (Bertolino *et al.*, 2019; Chen *et al.*, 2021). One of the main drivers of climate change is the increase in atmospheric CO_2 concentration, which has risen from ~280 ppm during the preindustrial period to >420 ppm today and is projected to keep rising in the coming decades (Cernusak *et al.*, 2013; Xu *et al.*, 2016; IPCC, 2023). Agriculture, forestry, land use and land use change are collectively reported to contribute up to 22 % of global greenhouse gas emissions. However, their contributions vary substantially between regions in terms of total magnitude. Tropical forests, which are an important buffer against climate change, are susceptible to current and future climate variations, temperature extremes, prolonged drought periods, heavy precipitation, tropical cyclones and other climate-extreme events (Crous *et al.*, 2025). The structure, functioning and productivity of tropical forests have probably already been affected by climate change, with evidence suggesting that CO_2 fertilization has enhanced gross and net primary productivity (Lewis *et al.*, 2009*a*, *b*), the tropical forest carbon sink already having peaked (Hubau *et al.*, 2020).

Studying the impact of climate change on individual species can help us better understand and predict overall patterns of change in the productivity of tropical rainforests due to altered climate conditions. Most studies on plant responses to climate change in the tropics have focused on canopy trees (e.g. Bonal *et al.*, 2011; Bauters *et al.*, 2020). However, much less is known about woody understorey species, which limits our understanding of the overall impacts of climate change on tropical forest systems (Fichtler *et al.*, 2010; Van Der Sleen *et al.*, 2017; Hatangi *et al.*, 2023). Santiago and Wright (2007), for example, showed that functional relationships of photosynthetic capacity vary among growth forms and depend on microclimatic conditions. Forest understorey species are exposed not only to lower light intensities but also to higher CO_2 concentrations compared with canopy trees (Hubau *et al.*, 2019; Damasceno *et al.*, 2024). Woody understorey species are now believed to be more resilient to drought and warming in tropical forests than previously expected (Alonso-Rodríguez *et al.*, 2022). Given that canopy tree results cannot be simply extrapolated to understorey species, targeted research is urgently needed to understand the impacts of climate change on this critical component of tropical biodiversity. Here, we focus on the most well-known and valuable understorey species in the Congo Basin rainforest, *Coffea canephora*, the wild predecessor of the cultivated Robusta coffee. It is a shrub that grows up to 12 m high and is widely, naturally distributed in the understorey of tropical lowland rainforests in Central and West Africa (Davis *et al.*, 2006). Recent studies have shown that considerable agronomic (Kiwuka *et al.*, 2021; Verleysen *et al.*, 2023) and organoleptic (Bollen *et al.*, 2024) potential is still unlocked in wild populations.

Morphological and physiological leaf traits of tropical plants can provide valuable insight into the response of plants to environmental change, although they remain understudied in the tropics compared with temperate ecosystems (Visscher *et al.*, 2022). Among traits, specific leaf area (SLA) and intrinsic water use efficiency (iWUE) are particularly informative for assessing plant physiological responses, especially in the context of increasing atmospheric CO_2. An increase in iWUE is typically attributed to higher net photosynthesis (A) and reduced stomatal conductance (g_s), both of which are influenced by SLA (Belmecheri *et al.*, 2021; Mathias and Thomas, 2021). These responses, enhanced photosynthesis and reduced stomatal conductance (g_s), are the most documented plant reactions to elevated atmospheric CO_2 (Long *et al.*, 2004; Ainsworth and Rogers, 2007; but see Rakocevic *et al.* (2018) for adult *Coffea arabica*). Tropical woody species may be particularly sensitive to atmospheric CO_2 enrichment, due to enhanced suppression of photorespiration at higher temperatures (Cernusak *et al.*, 2013). Most studies have reported an increase in iWUE in tropical forests in response to rising atmospheric CO_2 (Nock *et al.*, 2011; Van der Sleen *et al.*, 2015; Brienen *et al.*, 2017), while others have shown no variation (Bonal *et al.*, 2011) or a decrease in iWUE (Huang *et al.*, 2016; Bauters *et al.*, 2020). A study focusing on five understorey species in the Congo Basin, including *C. canephora*, found that the leaves had become significantly larger and had a higher SLA in the past 60 years, likely in response to climate change (Hatangi *et al.*, 2023). However, very little is known about both spatial variation and century-long temporal shifts in leaf morphological traits and iWUE in tropical understorey species, limiting our ability to predict the impacts of climate change on this forest layer.

We aimed to address this knowledge gap by combining analyses of leaf morphological and physiological traits, namely SLA, stomatal size (S), stomatal pore size (SPS), stomatal density (SD), maximum diffusive stomatal conductance to CO_2 (g_{cmax}), stable isotopes ($\delta^{13}C$ and $\delta^{18}O$) and iWUE from herbarium specimens collected over the past century, with a broad sampling throughout the Democratic Republic of the Congo (DRC) for the economically important understorey shrub *C. canephora*. This unique set-up enabled us to address the following questions: (1) Have the leaf traits and iWUE of *C. canephora* changed significantly over the past century? (2) Are these temporal changes consistent across different sites? (3) Do changes in leaf morphological traits explain the variation in iWUE? (4) Can spatial and temporal leaf traits and the iWUE of *C. canephora* variation be related to environmental gradients in the Congo Basin?

We hypothesize that (1) SLA and iWUE have increased over time, while SD and g_{cmax} have decreased; (2) these changes are consistent between sites in forests of the Congo Basin forests; (3) variation in leaf morphology explains the difference in iWUE; and (4) leaf morphological and physiological traits and iWUE of *C. canephora* relate to environmental variation throughout the Congo Basin.

MATERIALS AND METHODS

Sampling design and leaves conservation

Data were collected from 179 *Coffea canephora* herbarium specimens collected in the DRC and kept in the Herbarium of Meise Botanic Garden (BR) in Belgium (Supplementary Data Table S1). Our data set included 89 'old' herbarium specimens collected throughout the DRC between 1900 and 1960, and 90 'recent' herbarium samples collected in four provinces (Tshopo, Ituri, Sankuru and Bas-Uéle) between 2016 and 2021 (Fig. 1). Very few samples were collected between 1960 and 2016, and these were therefore not included in the study. We analysed the Climatic Research Unit (CRU) Time-Series (TS) version 4.09 dataset (Harris *et al.*, 2020) for historical air temperature (Supplementary Data Fig. S1) and precipitation (Supplementary Data Fig. S2) using the ncdf4 package (v.1.24; Pierce, 2025) in R (v.4.4.2; R Core Team, 2025) to characterize the main sampling sites. The leaves of recent specimens were collected on shrubs of *C. canephora* with a height of 0.5 and 10 m. The leaves of old herbarium specimens were collected mainly from shrubs that carried fruits or flowers, as botanical collectors preferred these. In the understorey of the wet forest area, *C. canephora* shrubs usually flower when 5–10 m high (H. Yves, pers. obs.). Subsequently, all dried leaves were dried at room temperature for 48 h to standardize tissue condition in all samples.

Leaf morphological traits measurements

For each specimen, three replicate leaves were used for measurements of leaf morphological traits. Stomatal density and

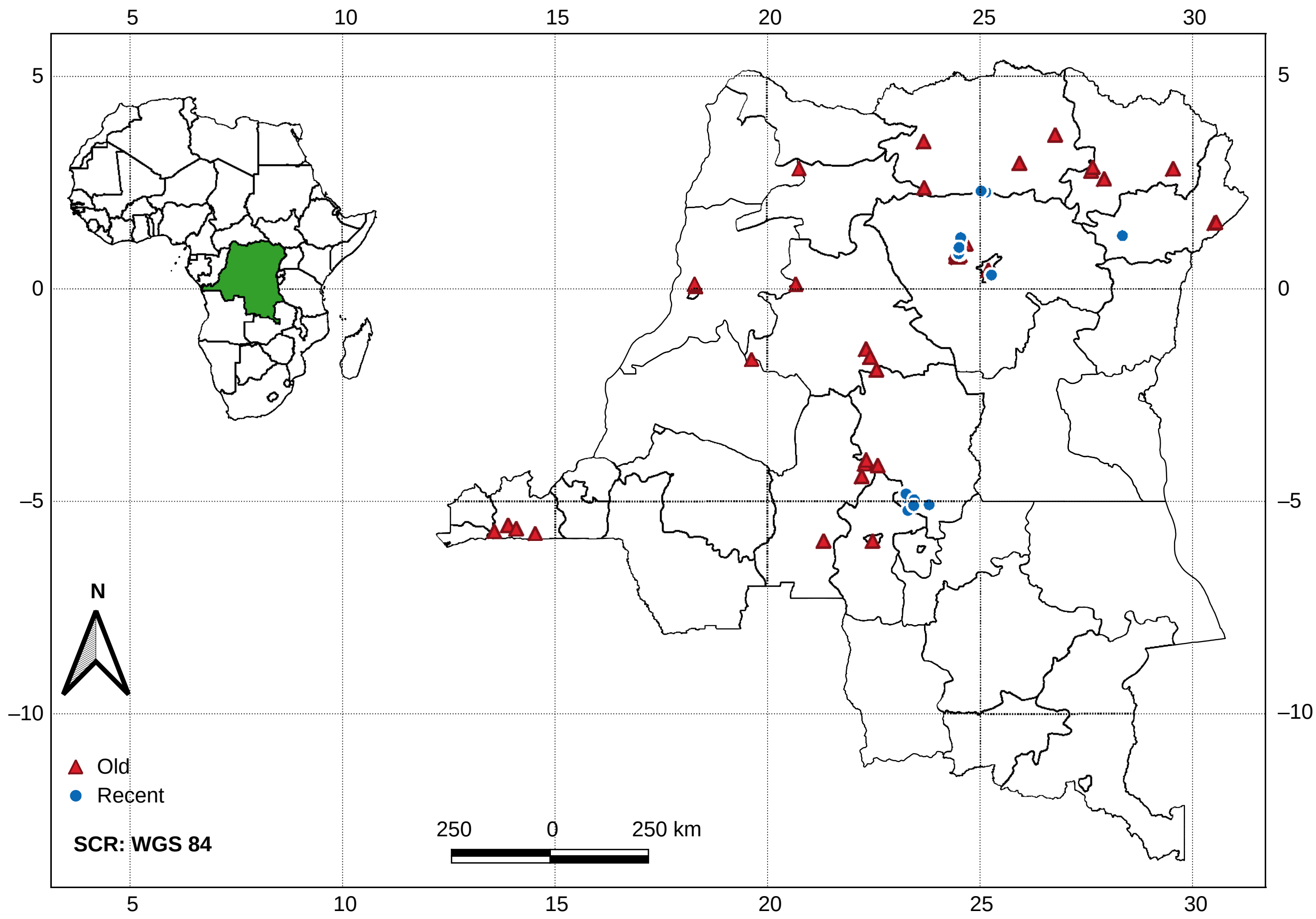

Fig. 1. Location of old (red triangles) and recent (blue circles) herbarium samples collected in the DRC and used in this study. Old herbarium samples were collected between 1900 and 1960 in the understorey of intact forests at the time of collection, but some of these forests have now disappeared. Recent samples were collected between 2016 and 2021 in Congolese provinces where there are still intact forests today.

stomatal size were obtained by making microscopic impressions of the abaxial side of all three leaves, using the collodion method (Stojnić *et al.*, 2015) by applying colourless nail varnish on both sides of the main vein. After 12–16 h, the varnish was removed using adhesive tape and placed on microscope slides following Meeus *et al.* (2020). Using a digital microscope (VH-5000 v.1.5.1.1, Keyence Corporation, Osaka, Japan), we made four photomicrographs of 1600 × 1200 pixels per leaf imprint (dimensions 344 × 258 µm) under a field of view of 0.09 mm^2 with full coaxial illumination and with default factory settings for shutter speed at an objective magnification of ×1000 (VH-Z250R). Stomata were manually counted using ImageJ v.1.52a software (US National Institutes of Health; https://imagej.net/ij/) within a grid of 40 000 $\mu\mathrm{m}^2$, following Hatangi *et al.* (2023). In each photo, the length (L) and width (W) of a stomata were measured and the surface area was calculated using the formula πab, where $a = ½L$ and $b = ½W$, assuming an ellipsoidal shape (Bonal *et al.*, 2011; Ramalho *et al.*, 2013; Rodrigues *et al.*, 2016). Stomatal pore size was calculated similarly using pore length and width.

The maximum diffusive stomatal conductance to water vapour (g_{wmax}, mol $\mathrm{m}^{-2}\ \mathrm{s}^{-1}$) was calculated according to Franks and Beerling (2009), as follows:

$$g_{w\,\max} = \frac{d}{v}.\mathrm{SD}.a_{\max}/\left(W + \frac{\pi}{2}\sqrt{\frac{a_{\max}}{\pi}}\right) \tag{1}$$

where d is the diffusivity of water vapour in air at 25 °C (0.0000249 $\mathrm{m}^2\ \mathrm{s}^{-1}$), v is the molar volume of air (0.0245 $\mathrm{m}^3\ \mathrm{mol}^{-1}$), $a_{\max}$ is the maximum area of the open stomatal pore ($\mu\mathrm{m}^2$), W is pore depth (approximated by the width of the stomata (µm)), and SD is stomatal density (mm^{-2}). Then, the maximum diffusive stomatal conductance to CO_2 (g_{cmax}) was calculated as follows:

$$g_{c\max} = g_{w\max}/1.6 \tag{2}$$

We used factor 1.6 as it is commonly accepted to represent the relationship between the maximum diffusive stomatal conductance to water vapour and the maximum diffusive stomatal conductance to CO_2 (Farquhar and Sharkey, 1982).

Finally, SLA was estimated following Pérez-Harguindeguy *et al.* (2013). On each leaf, a circular disc of 4.9 cm^2 (2.5 cm diameter) was punched from the widest central part of the lamina, carefully avoiding the main vein. The discs were then weighed with a precision balance to determine their dry mass. The SLA values were corrected using the linear model $y = 1.2x \pm$

−4.1 ($R^2 = 0.97$) proposed by Perez *et al.* (2020), which allows adjustment of disc-based estimates to approximate the values obtained from the measurements of the area of the fresh leaves.

iWUE assessment

Stable carbon isotope composition derived from leaf cellulose ($\delta^{13}C$) was used to estimate the changes in iWUE. During the photosynthesis process, when carbon is fixed, the heavier stable isotope $\delta^{13}C$ is naturally discriminated against. At that time, plants contain a smaller ratio of $\delta^{13}C$ to $\delta^{12}C$ than does the CO_2 of the air that feeds them (Farquhar *et al.*, 1982; Farquhar and Richards, 1984; Rumman *et al.*, 2018). The method allows retrospective inference of past environmental conditions using archived herbarium material (Bauters *et al.*, 2020). Isotope analysis for iWUE assessment was performed at the Isotope Bioscience Laboratory (ISOFYS) of Ghent University (Belgium).

Cellulose was extracted using the Jayme–Wise method for wood, as described by Andreu-Hayles *et al.* (2019). This procedure removes proteins, lipids and other labile compounds, yielding a stable carbohydrate fraction that provides more reliable isotopic signals than bulk tissue and is preferred in herbarium studies because it preserves isotopic fidelity over long-term storage (Greer *et al.*, 2018). The three leaf discs per sample, previously punched for SLA measurement, were homogenized. The samples were placed in Teflon tubes attached to cellulose extraction polytetrafluoroethylene (PTFE) devices. Four PTFE devices (95 samples total) were submerged in a Grant Sub Aqua 34 Plus digital water bath at 80 °C. The samples were rinsed three times with Milli-Q water heated to 100 °C and drained using a pump. A 0.158 M $NaClO_2$ solution was then added and the samples were left for 1 h. The acid treatment was repeated six times with reactivations every hour. The samples were rinsed three times with hot Milli-Q water, and as the bath cooled to 70 °C additional rinses with Milli-Q at room temperature were performed to stabilize the pH. At 70 °C, 2.50 M NaOH was added for 45 min, then drained, followed by three Milli-Q rinses at room temperature. The samples were soaked in water for 12 h to complete cellulose extraction. A second 45-min treatment with 2.50 M NaOH was applied at room temperature, followed by three rinses. Finally, the digital water bath was reheated to 80 °C and two 1-h treatments with 0.158 M $NaClO_2$ were carried out, followed by five final Milli-Q rinses. The extracted cellulose was stored in Eppendorf tubes for further analysis.

Before weighing, the cellulose samples were frozen at −50 °C for at least 24 h to remove residual moisture. For $\delta^{13}C$ analysis, 0.8 ± 10 % mg of cellulose was weighed using an ultramicrobalance (Radwag, readability 0.1 µg, Radom, Poland) and sealed in 8 × 5 mm tin capsules (Elemental Microanalysis, Okehampton, UK). The capsules were tightly closed and compressed before analysis by EA-IRMS (IsoLink Elemental Analyzer interfaced via ConFlo IV to a Delta Q Isotope Ratio Mass Spectrometer, Thermo Scientific, Bremen, Germany). The $\delta^{13}C$ values were normalized to the V-PDB scale using acetanilide (−29.50 ± 0.02 ‰) and caffeine (−1.17 ± 0.04 ‰) as calibration standards. IAEA cellulose (−24.72 ± 0.12 ‰) served as a quality control standard. For $\delta^{18}O$ analysis, 0.8–1 mg of cellulose was weighed and placed in 6 × 4 mm silver capsules (Elemental Microanalysis, Okehampton, UK). The capsules were unsealed and oven-dried for at least 5 d prior to measurement. The samples were analysed using a high-temperature thermal conversion element analyser (TC/EA, SerCon, Crewe, UK) equipped with a ceramic tube containing glassy carbon chips and a molybdenum liner, coupled to a 20–20 isotope ratio mass spectrometer (IRMS; SerCon). The $\delta^{18}O$ values were normalized to the V-SMOW scale using USGS90 (millet flour, 35.9 ± 0.29 ‰) and USGS91 (rice flour, 21.13 ± 0.44 ‰) as calibration standards. IAEA cellulose (32.52 ‰) was used as a quality control standard.

We derived historic $\delta^{13}C\text{-}CO_2$ ($\delta^{13}C_{atm}$) values from the equation in Bonal *et al.* (2011) for calculation of the iWUE. We used the classic model of carbon isotope discrimination during CO_2 fixation in the leaves of C3 plants to derive leaf discrimination against $\delta^{13}C$ in atmospheric CO_2 and $\delta^{13}C$ in cellulose ($\Delta^{13}C_{leaf}$) (Farquhar *et al.*, 1982) as:

$$\Delta^{13}\text{Cleaf} = \frac{\delta^{13}C_{\text{atm}}\ (‰) - \delta^{13}C_{\text{leaf}}(‰)}{1 + \delta^{13}\text{Cleaf}(‰)/1000} \tag{3}$$

Then we calculated the mole fractions of CO_2 in the intercellular spaces of the leaf (C_i) as follows:

$$C_i = \frac{C_{\text{atm}}\ (\Delta^{13}C_{\text{leaf}} - a) + f\Gamma*}{b\ -\ a} \tag{4}$$

where a is the isotope fraction during CO_2 diffusion through the stomata ($a = -4.4$ ‰), b is the isotope fraction caused by Rubisco and phosphoenolpyruvate carboxylase ($b = -27$ ‰) and $f\Gamma^*$ is the isotope fractionation through photorespiration (with $f = 12$ ‰ and Γ^* the CO_2 compensation point in the absence of day respiration ≈ 40 ppmv) according to Farquhar *et al.* (1982) and Bauters *et al.* (2020).

We defined iWUE defined as the ratio of photosynthesis, which is the net CO_2 assimilation rate (A_{net}) to stomatal conductance (g_s), and can be calculated as follows:

$$\text{iWUE} = \frac{A_{\text{net}}}{g_s} = \frac{C_{\text{atm}}}{1.6}\left(1 - \frac{C_i}{C_{\text{atm}}}\right) \tag{5}$$

Environmental data acquisition

Using the geographical coordinates provided in the herbarium metadata (Supplementary Data Table S1), we extracted climate data for each leaf sampling site from the WorldClim 2.1 database (https://www.worldclim.org/; accessed 12 August 2024). WorldClim provides long-term average climate data extrapolated from weather stations for the period 1970–2000. Data were accessed via DIVA-GIS 7.5.0 (Hijmans *et al.*, 2001). To these climate variables we added historical atmospheric CO_2 concentrations (C_{atm}) prior to 1960 using data from Van der Sleen *et al.* (2015), which are based on the Mauna Loa CO_2 record (Keeling *et al.*, 2005; https://scrippsco2.ucsd.edu/; accessed 14 August 2024). For 2020 and 2021, C_{atm} values were compared with field measurements from an eddy-covariance (EC) tower at the CongFlux site in Yangambi, DRC (Sibret *et al.*, 2022).

Data analysis

All analyses were conducted in R (v.4.4.2; R Core Team, 2025), with significance thresholds set at $P < 0.05$. Before statistical modelling, we tested the normality of all leaf traits using the Shapiro–Wilk test and assessed homoscedasticity between sampling sites using the Bartlett test, to verify ANOVA assumptions. Based on these results, all functional trait values were logarithmically transformed for subsequent modelling.

To assess long-term trends in *C. canephora* leaf traits over the past century, we fitted linear mixed effects models (LMMs) with the herbarium sampling year as a fixed effect and region and herbarium specimen as random effects. To investigate spatial variation during the two study periods (old, 1900–60; recent, 2016–21), the LMMs were also fitted with the sampling region and the period as fixed effects and the herbarium specimen as a random effect. Sampling sites from the same geographical area were grouped into three regions based on climatic similarity (mainly temperature and precipitation): (1) Kasaï region (Kasaï and Sankuru provinces), (2) Tshopo region (Yangambi, Ngazi, and Yoko sites), and (3) Uélé region (Bas-Uélé, Haut-Uélé and Epulu forest in Ituri province). The relationships between leaf morphological traits and iWUE were evaluated using standardized major axis regression (SMA), implemented via the sma() function in the smatr package (v.3.4.8; Warton *et al.*, 2012). The analysis was performed on old and recent samples separately to avoid bias due to temporal variation. The effect of environmental variables on trait variation was tested by fitting additional LMMs with environmental drivers as fixed effects, and the collection site, year and herbarium sampling were replicated as random effects. To select the most relevant environmental variables, we performed a principal component analysis (PCA) that included all climatic, CO_2 and location data, using the PCA() function of the FactoMineR package (v.2.11; Lê *et al.*, 2008) Based on high eigenvalues, we retained five variables that contributed the most to overall variation: C_{atm}, mean annual temperature (MAT), temperature seasonality (TS), mean annual precipitation (MAP) and precipitation seasonality (PS). Temperature seasonality represents the variability of the temperature between years and was calculated as the standard deviation of monthly mean temperatures × 100. Precipitation seasonality reflects monthly rainfall variability and was expressed as the coefficient of variation (CV) of monthly precipitation totals, calculated as (standard deviation of monthly precipitation/mean monthly precipitation) × 100. All models were fitted using maximum likelihood with the lmer() function from the lme4 package (v.1.1-35.5; Bates *et al.*, 2015). The significance of fixed effects was evaluated using Wald's χ^2 tests via the anova() function of the lmerTest package (v.3.1.3; Kuznetsova *et al.*, 2017). The explanatory power of the model was assessed through marginal R^2 (variance explained by fixed effects) and conditional R^2 (total variance explained) using the r.squaredGLMM() function from the MuMIn package (v.1.48.4; Bartoń, 2024). The means and standard errors were grouped by region. All graphs were created using the ggplot2 package (v.3.5.1; Wickham, 2016) and arranged using the plot_annotation() function from the patchwork package (v1.10.3; Pedersen, 2024).

RESULTS

Temporal variation of the morphological traits of C. canephora *leaves and iWUE*

The leaf morphological traits of *C. canephora* exhibited significant long-term changes between 1900 and 2021. Specific leaf area increased significantly over time (Table 1; $P < 0.001$). rising from 148.5 ± 4.4 cm^2 g^{-1} in 1900 to 200.9 ± 3.2 cm^2 g^{-1} in 2021 (+35 %; Fig. 2A). On the contrary, SD decreased significantly (Table 1; $P < 0.001$) from 408.3 ± 12.2 mm^{-2} in 1900 to 317.2 ± 5.2 mm^{-2} in 2021 (−22 %; Fig. 2D). No significant change in S was observed over time (Fig. 2B; Table 1; $P > 0.05$), and remained around 400 μm^2 on average. Stomatal pore size decreased significantly over time (Table 1; $P < 0.001$): pores were larger in 1900 (92.7 ± 2.8 μm^2) than in 2021 (78.3 ± 1.2 μm^2) (−16 %; Fig. 2C).

The explanatory power of the models varied by trait (Table 1). For SLA and SPS, the marginal R^2 (R^2_{m}) values were 0.29 and 0.16, respectively, indicating that the fixed effect of year explained, respectively, 29 and 16 % of the variation in these traits. Conditional R^2 (R^2_{c}) values for these traits were the same (0.51), reflecting the added contribution of random effects such as variation between regions, individuals and the replicate of the herbarium. Stomatal density exhibited slightly lower explanatory power ($R^2_{\text{m}} = 0.06$; $R^2_{\text{c}} = 0.54$) and S showed very low values ($R^2_{\text{m}} = 0.018$; $R^2_{\text{c}} = 0.48$), consistent with the lack of a significant temporal trend. These R^2 values emphasize that while year is an important driver of variation in key leaf traits, additional sources of variation must be considered to fully understand the dynamics of traits over time.

The physiological characteristics of the leaves of *C. canephora* showed clear long-term trends over the past century (Table 1, Fig. 3). The δ^{13}C values of leaf cellulose values showed a strong and significant decline, from -27.8 ± 0.3 ‰ in the early 1900s to -32.5 ± 0.2 ‰ in 2021 (Fig. 3A; $R^2_{\text{m}} = 0.53$, $R^2_{\text{c}} = 0.84$, $P < 0.001$). This trend is consistent with the progressive depletion of atmospheric δ^{13}C and also indicates pronounced changes in the dynamics of carbon within the plant. In contrast, δ^{18}O values remained relatively constant throughout the same period, with a mean of 29.7 ± 0.1 ‰ and no significant temporal trend (Fig. 3B; $R^2_{\text{m}} = 0.001$, $R^2_{\text{c}} = 0.03$, not significant).

During the past century, a marked decline was observed in g_{cmax}, which decreased from 1.1 ± 0.1 mol m^{-2} s^{-1} in 1900 to 0.7 ± 0.02 mol m^{-2} s^{-1} in 2021 (Fig. 3C; $R^2_{\text{m}} = 0.22$, $R^2_{\text{c}} = 0.53$, $P < 0.001$). The intercellular CO_2 concentration (C_{i}) increased substantially, from 228.0 ± 4.9 to 355.7 ± 4.5 μmol mol^{-1} (data not shown). These coordinated changes suggest reduced g_{s} and increased CO_2 availability within the leaf, which could reflect long-term acclimatization to elevated atmospheric CO_2. Due to these changes, iWUE decreased significantly over time, from 43.5 ± 3.1 μmol mol^{-1} in 1900 to 35.2 ± 1.7 μmol mol^{-1} in 2021 (Fig. 3D; $R^2_{\text{m}} = 0.04$, $R^2_{\text{c}} = 0.67$, $P < 0.001$), indicating reduced efficiency in balancing carbon assimilation against water loss under historical and contemporary environmental conditions. For isotopes and g_{cmax}, R^2_{m} showed that year explained a substantial part of the variance (0.36 for δ^{13}C and 0.16 for g_{cmax}; Table 1). The R^2_{c}, which includes regional and herbarium replicate effects, was higher (0.91 and 0.46, respectively), indicating that both temporal and spatial factors influence trait variation. For iWUE, year explained a

TABLE 1. Results of linear mixed effect models that examine variation in leaf morphological and physiological traits, as well as intrinsic water use efficiency (iWUE), of *Coffea canephora* over time in Congo Basin forests.

Traits	SLA	S	SPS	SD	g_{cmax}	δ^{13}C	δ^{18}O	iWUE
Fixed effect								
Intercept	2.69	6.92	8.91	6.77	4.85	48.87	3.4	7.8
Coefficient (year)	0.001	−0.0005	−0.002	−0.00046	−0.002	−0.04	−0. 00007	−0.002
F-value (year)	33.41***	15.25ns	51.44 ***	3.74*	85.39***	39.73***	0.08ns	0.04***
Model fit								
R^2_{m}	0.29	0.018	0.16	0.06	0.22	0.53	0.001	0.04
R^2_{c}	0.51	0.48	0.51	0.54	0.53	0.84	0.03	0.67

Fixed effects include the intercept and the coefficient for year.

The *F*-values are from type III ANOVA with the Satterthwaite method, and the significance levels are indicated as follows: ns, not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Models were evaluated based on marginal R^2 (R^2_{m}), representing the proportion of variance explained by fixed effects (i.e. year) and conditional R^2 (R^2C), which accounts for both fixed and random effects (including sampling region and herbarium replicate).

SLA, specific leaf area; S, stomatal size; SPS, stomatal pore size; SD, stomatal density; g_{cmax}, maximum diffusive stomatal conductance to CO_2; δ^{13}C, stable carbon isotope composition; δ^{18}O, stable oxygen isotope composition; iWUE, intrinsic water use efficiency.

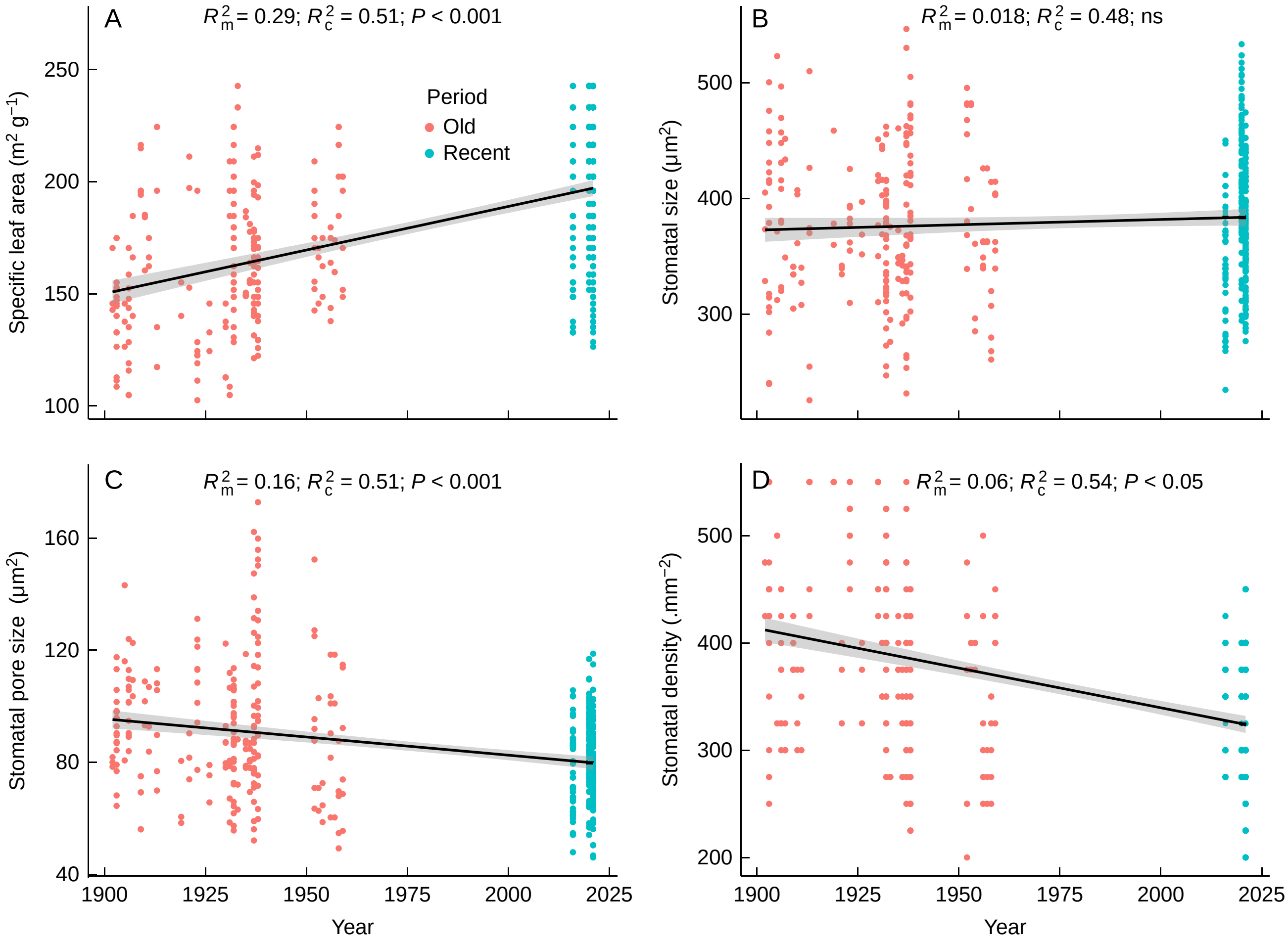

FIG. 2. Temporal trends in *Coffea canephora* leaf morphological characteristics in forests of the Congo Basin based on linear regression models. The panels show results from linear regression models fitted across samples collected between 1900 and 2021. (A) Specific leaf area; (B) stomatal size; (C) stomatal pore size and (D) stomatal density. Each point represents an individual herbarium specimen, coloured by collection period (red, old; blue, recent). The shaded areas indicate 95 % confidence intervals around the fitted regression lines. ns, not significant. R^2_{c} (conditional R^2) is total variance explained by both fixed and random effects (including sampling region); R^2_{m} (marginal R^2) is variance explained by fixed effects.

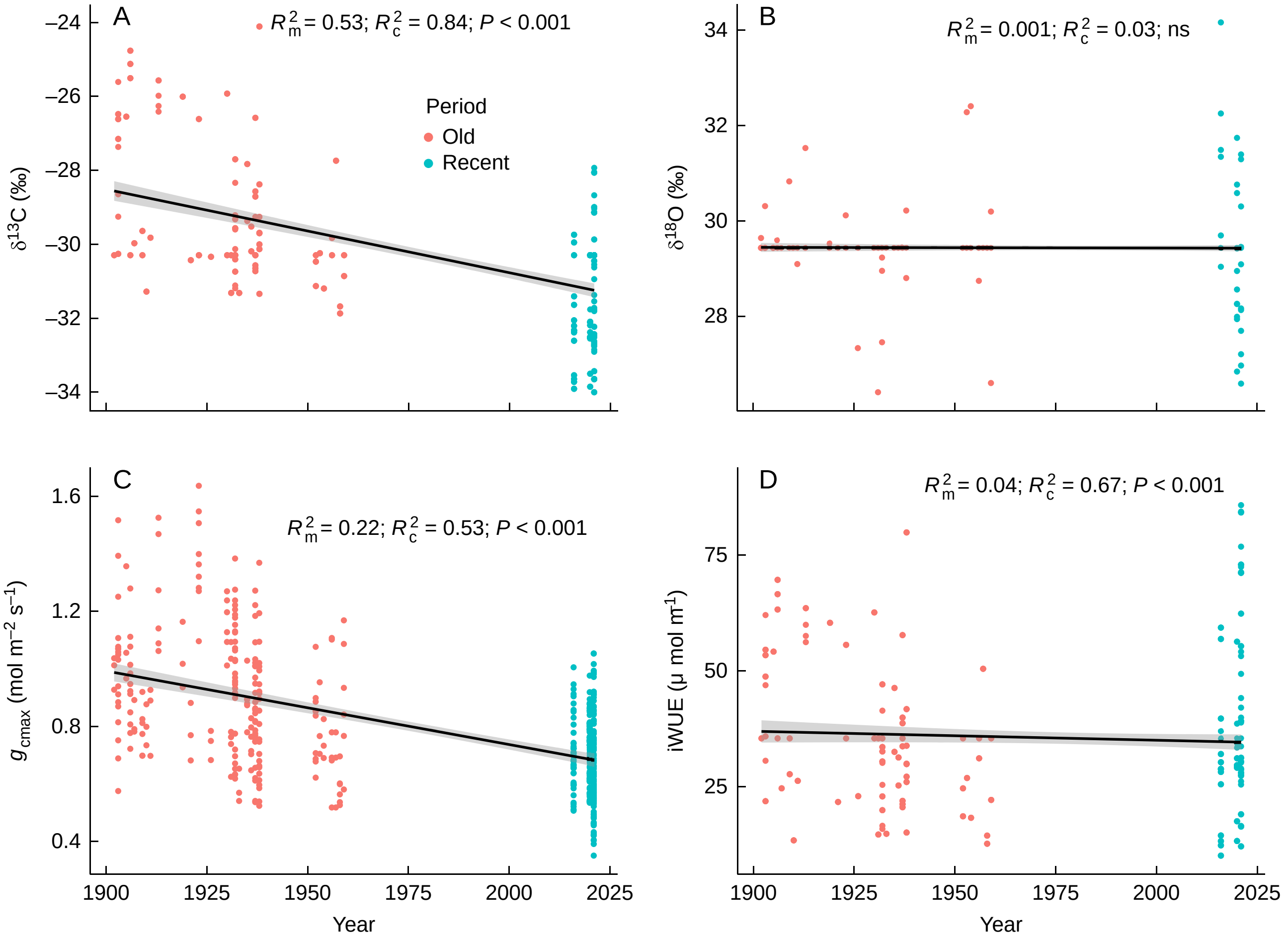

FIG. 3. Temporal trends in stable isotopic composition ($\delta^{13}C$, $\delta^{18}O$), maximum diffusive stomatal conductance to CO_2 (g_{cmax}) and intrinsic water use efficiency (iWUE) in *Coffea canephora* from forests in the Congo Basin. Panels show results from linear regression models fitted across samples collected between 1900 and 2021. (A) $\delta^{13}C$; (B) $\delta^{18}O$; (C) g_{cmax}; (D) iWUE. Each point represents an individual herbarium specimen, coloured by collection period (red, old; blue, recent). Shaded areas indicate 95 % confidence intervals around the fitted regression lines.

moderate amount of variance with a R^2_m of 0.00004, while the R^2_c was 0.87, indicating that both temporal trends and regional differences contribute to its variation. Effects of interactions between sampling year and region on *C. canephora* leaf traits are shown in Supplementary Data Table S2.

Spatial and temporal variation of morphological traits of the leaves of C. canephora *and iWUE*

The LMMs revealed both spatial and temporal variation of *C. canephora* leaf traits (Table 2, Fig. 4). The SLA differed significantly between time periods ($P < 0.05$) and between regions ($P < 0.001$). An overall increase in SLA was observed over time (Fig. 4A), with the highest values found in recent specimens from the Tshopo region. The size of the stomata did not differ significantly between the two collection periods but varied significantly between regions ($P < 0.05$), with the largest stomata observed in the Tshopo region (Fig. 4B). Stomatal pore size showed significant effects of both region and period ($P < 0.001$), with the highest values found in older Tshopo samples and a significant decrease over time (Fig. 4C). Stomatal density decreased significantly over time in the Kasaï and Uélé regions ($P < 0.001$; Fig. 4D).

The $\delta^{13}C$ values of *C. canephora* leaves differed significantly between the sampling regions and the collection periods, with significant interactions between sampling regions and collection periods for SLA, SD, g_{cmax}, $\delta^{13}C$ and C_i, while other traits showed no significant interactions (Table 2; $P < 0.001$). A temporal decrease in $\delta^{13}C$ was observed in all regions: from −27.2 ± 0.3 to −31.2 ± 0.3 ‰ in Kasaï, from −29.1 ± 0.2 to −33.1 ± 0.1 ‰ in Tshopo, and from −30.1 ± 0.4 to −32.7 ± 0.2 ‰ in Uélé (Fig. 5A). In contrast, $\delta^{18}O$ values did not show significant variation between regions or over time (Table 2). Across all samples, $\delta^{18}O$ values ranged from a minimum of 26.6 to a maximum of 34.2, with a mean of 29.6 ± 0.1 ‰. The concentration of intercellular CO_2 (C_i) increased significantly over time, with strong differences between both regions and periods (Table 2; $P < 0.001$). From 1900 to 2021, C_i rose from 220.7 ± 4.2 to 343.1 ± 5.9 µmol mol^{-1} in Kasaï, from 253.0 ± 3. to 377.9 ± 2.8 µmol mol^{-1} in Tshopo, and from 265.4 ± 6.3 to 366.9 ± 3.9 µmol mol^{-1} in Uélé (Fig. 5B). The g_{cmax} also decreased significantly over time and varied between regions (Table 2; $P < 0.05$). A decrease from 1.1 ± 0.1 to 0.8 mol m^{-2} s^{-1} was observed in Kasaï, from 0.8 to 0.7 mol m^{-2} s^{-1} in Tshopo, and from 0.9 to 0.6 mol m^{-2} s^{-1} in Uélé (Fig. 5C). Finally, iWUE decreased over time and differed significantly between regions

TABLE 2. Summary of results of LMMs analysing the effects of region and period on leaf morphological and physiological traits.

Trait	SLA	S	SPS	SD	g_{cmax}	$\Delta^{18}O$	$\Delta^{13}C$	C_i	iWUE
Fixed effects									
Intercept	5***	5.9***	4.48***	5.97***	−0.1ns	3.4***	−27.22***	5.53***	3.96***
Region	7.38***	3.75*	6.46*	8.84***	7.83***	4.9ns	21.32***	19.15***	23.32***
Period	3.34*	0.86ns	3.98*	0.63ns	4.11*	2.54ns	13.88**	47.99**	0.03ns
Region × period	1.11**	0.62ns	0.053ns	8.98**	4.65 *	0.05ns	0.13**	0.11**	0.43ns
Model fit									
R^2_m	0.18	0.05	0.18	0.09	0.18	0.29	0.47	0.7	0.25
R^2_c	0.73	0.48	0.61	0.65	0.65	0.89	0.95	0.96	0.97

Each model includes random intercepts for year and herbarium replicates. The table reports the estimated intercepts and *F*-values for each fixed effect, along with R^2_m and R^2_c values.

ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

SLA, specific leaf area; S, stomatal size; SPS, stomatal pore size; SD, stomatal density; g_{cmax}, maximum diffusive stomatal conductance to CO_2; $\delta^{13}C$, stable carbon isotope; $\delta^{18}C$, stable oxygen isotope; iWUE, intrinsic water use efficiency.

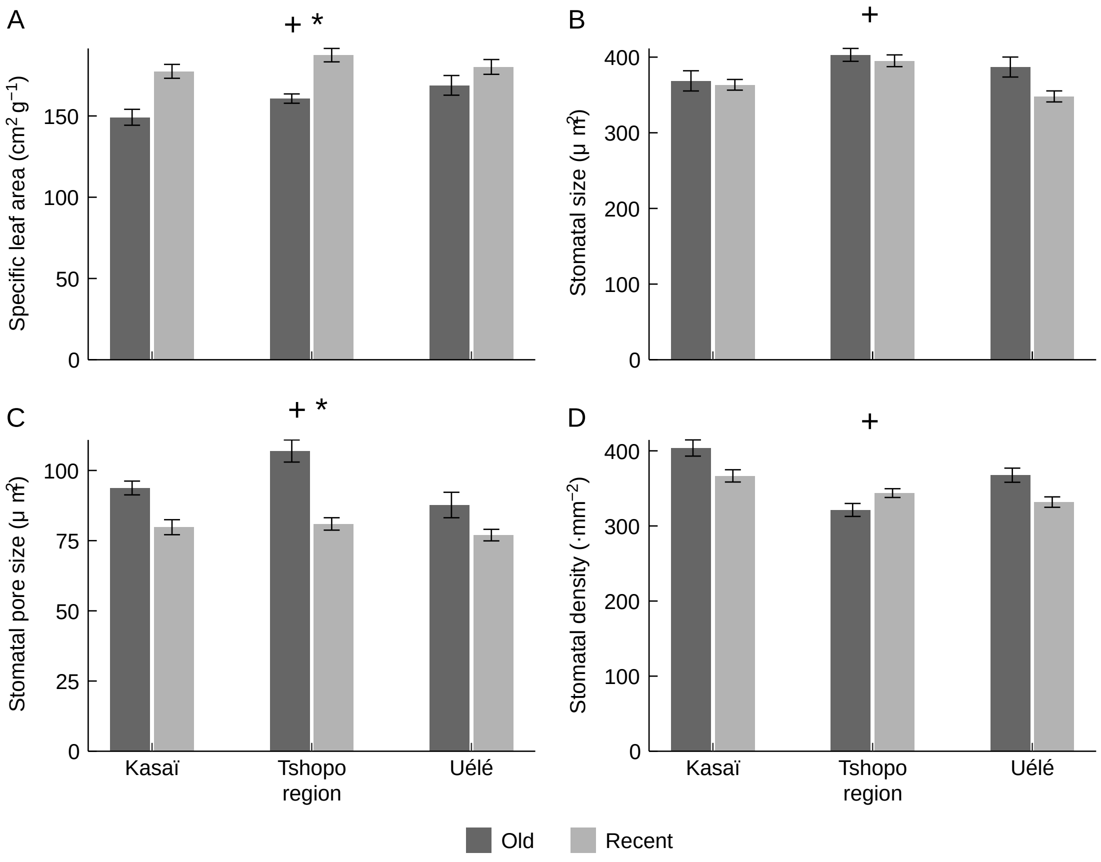

FIG. 4. Temporal and spatial variation in leaf morphological traits of *Coffea canephora* from three forest regions in the Congo Basin. Bars represent means and error bars denote standard errors. Symbols indicate statistically significant effects based on LMMs: + indicates a significant effect of region and * indicates a significant effect of collection period (old versus recent).

(Table 2; $P < 0.001$). iWUE dropped from 49.2 ± 2.7 to 45.8 ± 3.7 μmol mol^{-1} in Kasaï, from 35.3 ± 2.1 to 23.1 ± 1.7 μmol mol^{-1} in Tshopo, and from 26.0 ± 3.3 to 25.3 ± 2.3 μmol mol^{-1} in Uélé (Fig. 5D). More details on effects of sampling regions and collection period on *C. canephora* trait variation are given in Supplementary Data Table S3.

Relationship between C. canephora *leaf morphological traits and iWUE in the Congo Basin forests*

The SMA regression revealed significant relationships between iWUE and several key leaf traits. A strong negative relationship was observed between iWUE and SLA (Fig. 6A; $R^2 = 0.38$, $P < 0.001$), indicating that trees with higher SLA tend to

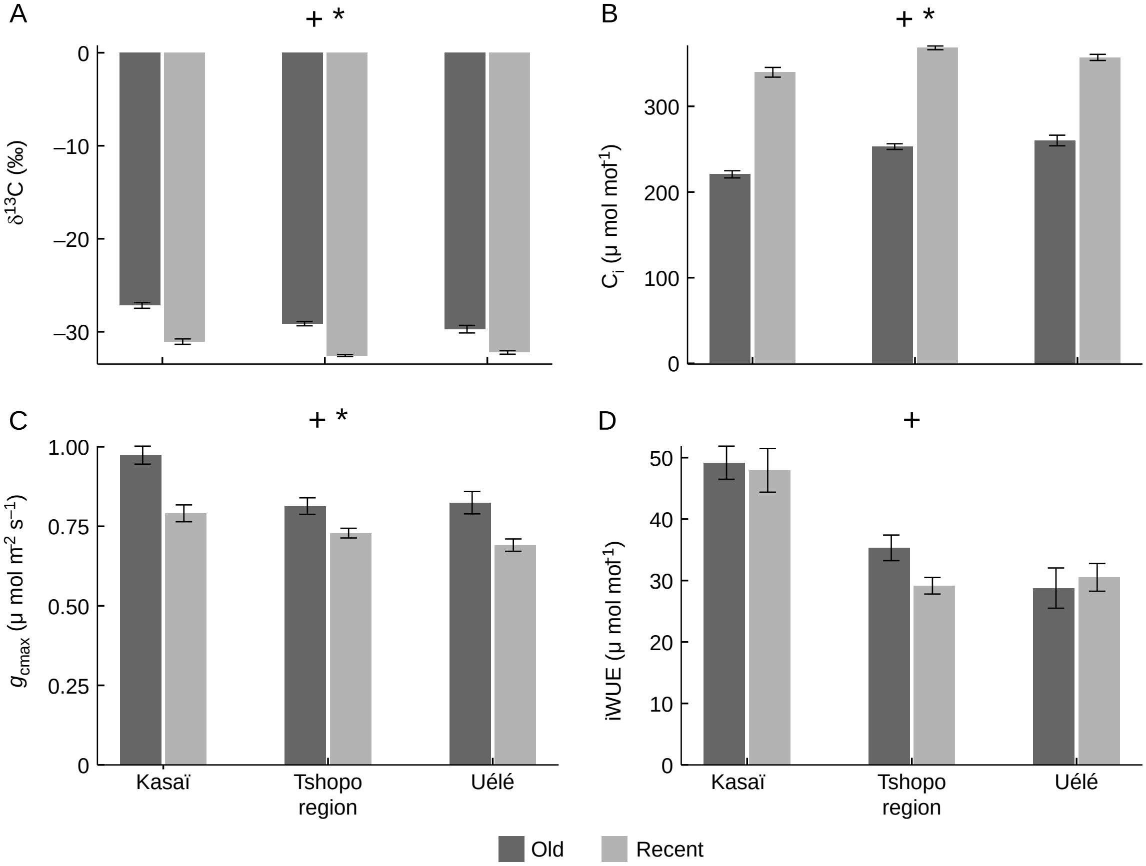

Fig. 5. Temporal and spatial variation in the physiological traits of *Coffea canephora* leaves from three forest regions in the Congo Basin. Bars represent means and error bars denote standard errors. Symbols indicate statistically significant effects based on LMMs: + indicates a significant effect of region and * indicates a significant effect of collection period (old versus recent).

have lower water use efficiency, highlighting a trade-off between leaf morphology and physiological performance. Furthermore, iWUE showed a moderate positive association with SD (Fig. 6C; $R^2 = 0.18$, $P < 0.001$), suggesting that a higher SD contributes to increased water use efficiency. Stomatal size exhibited a weaker but significant positive relationship with iWUE (Fig. 6B; $R^2 = 0.043$, $P < 0.05$), indicating that larger stomata may also slightly improve water use efficiency. Furthermore, the g_{cmax} showed a moderate positive correlation with iWUE (Fig. 6D; $R^2 = 0.24$, $P < 0.001$), reinforcing the role of stomatal traits in the regulation of plant water use strategies. Collectively, these findings highlight the complex interplay between leaf structural and physiological traits in shaping water use efficiency and adaptive responses to environmental conditions. Similar relationships among iWUE and traits using recent specimens collected between 2016 and 2022 are shown in Supplementary Data Fig. S3.

Environmental drivers of leaf traits and iWUE in C. canephora *in forests in the Congo Basin*

Environmental and geographical variables significantly influenced key leaf traits of *C. canephora* throughout the Congo Basin (Table 3). Linear mixed effects models revealed that the leaf morphological and physiological traits of *C. canephora* respond differentially to environmental drivers. The specific leaf area increased significantly with the mean annual temperature ($F = 7.36$, $P = 0.01$), while the variables C_{atm}, latitude and precipitation had no significant effect on SLA. Stomatal size and pore size both showed significant positive relationships with latitude (stomatal size: $F = 4.99$, $P = 0.045$; pore size: $F = 4.58$, $P = 0.043$), with pore size also showing a marginal positive effect of MAP ($F = 3.15$, $P = 0.082$). Stomatal density was not significantly influenced by any predictors, although TS showed a marginal negative trend ($F = 2.88$, $P = 0.1$). Physiological traits were more strongly influenced by atmospheric CO_2: g_{cmax} decreased significantly with C_{atm} ($F = 4.70$, $P = 0.043$) and C_{i} increased significantly with C_{atm} ($F = 42.87$, $P < 0.001$), MAT ($F = 11.25$, $P = 0.001$) and MAP ($F = 8.89$, $P = 0.003$). Latitude, TS and PS generally had no significant effect on physiological traits. The R^2_{m} values ranged from 0.08 to 0.46, and the R^2_{c} values reached up to 0.69, reflecting substantial random effects of region and year. These results highlight that morphological traits respond primarily to temperature and latitude gradients, whereas physiological traits are more sensitive to atmospheric CO_2 and precipitation.

DISCUSSION

Our results provide novel insights into the temporal and spatial variation of the leaf morphological and physiological traits of *C. canephora* that grows in the understorey of the Congo

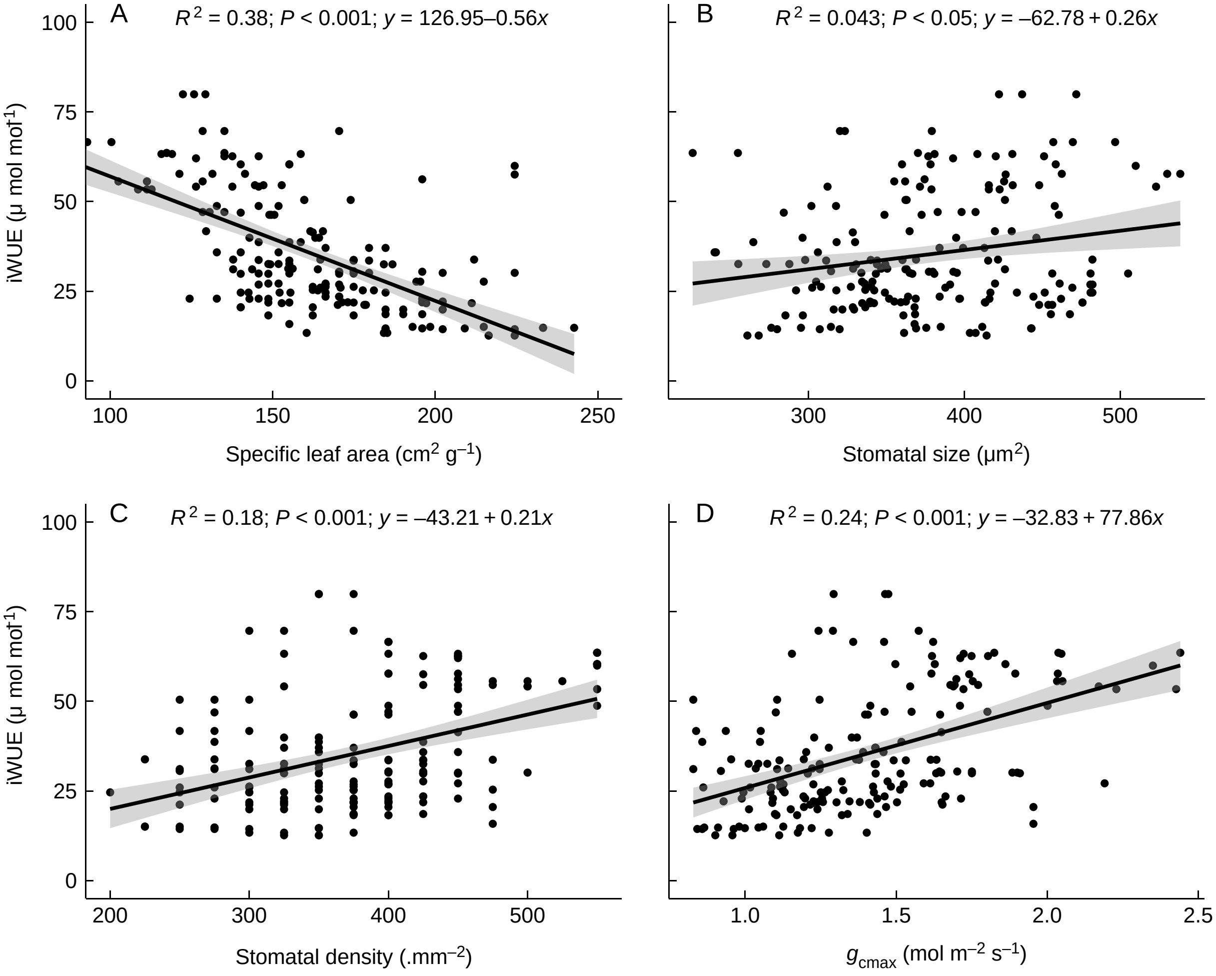

FIG. 6. Relationships between leaf morphological traits and intrinsic water use efficiency (iWUE) in *Coffea canephora* from forests of the Congo Basin, based on old herbarium samples collected between 1900 and 1960. Each panel shows a linear regression between iWUE and a morphological trait: (A) specific leaf area (SLA); (B) stomatal size; (C) stomatal density (SD); (D) maximum stomatal conductance to CO_2 (g_{cmax}). The shaded areas represent 95 % confidence intervals around the regression lines.

Basin rainforest. We observed an overall increase in SLA of *C. canephora* shrubs over the past century, while SD and iWUE decreased significantly.

However, the temporal changes were not consistent between regions. A significant interaction between the forest region and the collection period suggests that regional environmental differences modulate trait responses. The space-for-time approach did not hold fully in our study system, as the relationship between plant traits and environmental change did not always follow spatial correlations. Finally, contrary to theoretical expectations from the stomatal optimization theory, which predicts that iWUE should increase proportionally with increasing C_{atm} (Medlyn *et al.*, 2011; Gardner *et al.*, 2023), we observed a decline in iWUE that was not driven by increasing g_s. Instead, g_s also declined, suggesting that photosynthetic capacity may have diminished over time.

Long-term trends in functional leaf traits and iWUE

We observed considerable changes in the leaf morphological characteristics of *C. canephora* shrubs over time in forests of the Congo Basin. The increase in SLA we recorded from 1900 until 2021 is consistent with previous findings in five understorey woody species, one of which was also *C. canephora*, in Yangambi, DRC, over the same period (Hatangi *et al.*, 2023). The increase in SLA may be explained by the effect of CO_2 fertilization observed in tropical forests (Lewis *et al.*, 2009*a*, *b*; Hubau *et al.*, 2020; Wang *et al.*, 2024). Although understorey species generally have a low photosynthetic CO_2 assimilation rate per area and a high SLA compared with canopy trees (da Silveira *et al.*, 1989; Santiago and Wright, 2007), they do respond to atmospheric CO_2 enrichment by adjusting their main functional traits, especially their stomatal behaviour from intracellular signalling to whole-plant responses to global climatic change (Woodward, 1987; Woodward *et al.*, 2002; Hetherington and Woodward, 2003).

Our findings reveal significant temporal shifts in the physiological traits of *C. canephora* leaves, particularly a decline in foliar $\delta^{13}C$ values from the early 20th century to 2020–21. These more negative $\delta^{13}C$ values are consistent with the physiological responses expected in understorey species growing under low light conditions, where limitations in photosynthetic capacity lead to a decrease in iWUE (Fichtler *et al.*, 2010; Ge *et al.*, 2022). The range of $\delta^{13}C$ values observed in our study aligns closely with those reported for tropical understorey and shaded

TABLE 3. Fixed-effect coefficient estimates from LMMs that examine the influence of environmental variables on leaf morphological and physiological traits and iWUE of *C. canephora*.

Variable	SLA	S	PS	SD	g_{cmax}	C_i	iWUE
Intercept	5***	5.76***	4***	5.8***	1.2	4.22***	3.62**
C_{atm} (μmol mol^{-1})	0.007	0.0006	−0.0004	0.0002	0.0001	0.004***	0.005**
Latitude (°)	0.001	0.02 *	0.004*	−0.001	−0.01	−0.02*	−0.1**
MAT (°C)	0.0001 *	−0.0003	0.0004	−0.0005	0.0006	0.0002**	0.0006
TS (°C)	0.0001	−0.005	0.0005	−0.001	0.0002	0.001*	−0.006
MAP (mm)	−0.0001	−0.0009	0.0001	−0.0003	0.0001	−0.0005	−0.0009*
PS (mm)	−0.004	0.002	0.005	0.004	0.0006	−0.003	−0.001
Model fit							
R^2_m	0.19	0.09	0.13	0.21	0.19	0.4	0.2
R^2_c	0.65	0.58	0.66	0.75	0.68	0.9	0.82

The numbers are the coefficients of the LMM.

Marginal (R^2_m) and conditional (R^2_c) R^2 values represent the variance explained by fixed effects alone and by fixed and random effects, respectively.

The environmental variables used were atmospheric CO_2 concentration (C_{atm}), mean annual temperature (MAT), seasonal temperature (TS), mean annual precipitation (MAP) and seasonal precipitation (PS).

iWUE, intrinsic water use efficiency; SLA, specific leaf area; S, stomatal size; SPS, stomatal pore size; g_{cmax}, maximum diffusive stomatal conductance to CO_2; SD, stomatal density.

Only statistically significant coefficients ($P < 0.05$) are indicated with an asterisk: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

coffee plants, including *C. canephora* in Brazil (Peng *et al.*, 2019) and other Neotropical species (da Silveira *et al.*, 1989), supporting the ecological consistency of our data. The long-term decline in foliar $\delta^{13}C$ also mirrors the known decrease in atmospheric $\delta^{13}C$ ($\delta^{13}C_a$), largely attributed to fossil fuel combustion (the Suess effect), a trend that has been similarly documented in other tropical trees (Hietz *et al.*, 2005; Bonal *et al.*, 2011; Weiwei *et al.*, 2018; Bauters *et al.*, 2020). This suggests that *C. canephora* has experienced sustained physiological adjustments over time, which could reflect changes in carbon assimilation and stomatal regulation under evolving atmospheric conditions. Interestingly, the decline in $\delta^{13}C$ in *C. canephora* appears more pronounced than in some canopy species, for which foliar $\delta^{13}C$ has remained relatively stable despite the drop in $\delta^{13}C_{atm}$ (Nock *et al.*, 2011). This divergence may be attributed to ecological positioning and functional strategy: as an understorey shrub, *C. canephora* likely operates in a light- and carbon-limited environment and may be more sensitive to atmospheric changes, particularly when combined with stable light conditions and conservative water use strategies. Therefore, this observed isotopic trend may not only reflect atmospheric forcing, but also point to an interaction between global change and functional leaf traits under persistent microclimatic limitations. These findings underscore the utility of stable isotopes as retrospective indicators of physiological function and highlight the importance of considering forest strata and life history when evaluating plant responses to environmental change.

The observed increase in C_i for *C. canephora* shrubs was consistent with patterns reported for *Cedrela odorata* and *Swietenia macrophylla* trees in the Amazonian Basin (Hietz *et al.*, 2005) and for other tropical understorey and canopy tree species in Bolivia, Cameroon and Thailand under increasing [CO_2] and iWUE during the last century (Van der Sleen *et al.*, 2015). A decrease in $\delta^{13}C$ is generally interpreted as an increase in C_i, driven either by reduced g_s or enhanced photosynthetic capacity (Farquhar *et al.*, 1982; Scheidegger *et al.*, 2000). In our case, we observed a decrease in both $\delta^{13}C$ and g_{cmax}, suggesting that the increase in C_i was not driven by stomatal opening, but rather by a decrease in photosynthetic capacity, possibly in response to environmental change in the Congo Basin. In contrast to $\delta^{13}C$, we found no significant temporal trend in $\delta^{18}O$ values, consistent with results obtained by Bauters *et al.* (2020) for the Yangambi region. They proposed that the stability in $\delta^{18}O$ over time reflects limited changes in water availability, due to relatively constant precipitation patterns and high ambient humidity in tropical forests, which reduce the evaporative enrichment at the leaf level. Finally, we hypothesized an increase in SLA, S and iWUE over the past century, along with a decrease in SD and g_s. Our results only partially support this hypothesis: while SLA increased and both SD and g_s decreased, iWUE also declined. This contradicts theoretical expectations and several empirical studies showing an increase in iWUE in temperate and tropical forests due to elevated CO_2 (Saurer *et al.*, 2004; Hietz *et al.*, 2005; Van der Sleen *et al.*, 2015; Mathias and Thomas, 2021).

However, our findings are consistent with a recent study in Yangambi, DRC, which documented declining iWUE in 13 species (Bauters *et al.*, 2020), and with observations from tropical forests in China showing a 30 % decrease in iWUE from 1950 to 2014, attributed to reduced photosynthetic capacity, light limitation or mesophyll conductance (Huang *et al.*, 2016). It is well known that iWUE varies substantially with forest type, species, age, climate and management practices (Wang *et al.*, 2018; Ge *et al.*, 2022; Zhang *et al.*, 2023). While long-term iWUE increases are typically interpreted as evidence for reduced transpiration or enhanced photosynthesis (by decreasing g_s), it was paradoxical to observe through our data that iWUE decreased over time while stomatal conductance (g_{cmax}) and photosynthetic capacity decreased too, according to the conceptual model of Scheidegger *et al.* (2000). These results are similar to observations found by Crous *et al.* (2025), who mentioned that g_s and photosynthesis decreased with warming. Furthermore, it is known through previous studies that iWUE increases proportionally with increasing atmospheric carbon concentration, when the C_i/C_{atm} ratio remains relatively constant (Belmecheri *et al.*, 2021; Ma *et al.*, 2023). This situation challenges current knowledge of plant physiology, particularly in tropical ecosystems, where species often exhibit responses not captured by temperate-based models. It is not entirely clear whether the effect of the increase in atmospheric CO_2 concentration is apparent in the forests of the Congo Basin, contrary to the results previously obtained for temperate tree species and *Coffea arabica* from the free air CO_2 enrichment (FACE) experiments, which showed increased photosynthesis and decreased transpiration (Leakey *et al.*, 2009; Norby and Zak, 2011; Ghini *et al.*, 2015; Rakocevic *et al.*, 2018). The results of this study challenge the presumed resilience of *C. canephora* trees to climate change in the Congo Basin forests. An increase in S and decreases in SD, g_{cmax} and iWUE over time have been found. The decrease in iWUE over time may probably result from decreasing photosynthetic capacity (Grams *et al.*, 2007; Huang *et al.*, 2016). It is generally known that plants with high SD and smaller S (resulting in higher g_{smax} and g_{cmax}) are more resilient to drought and climate change than low SD and large stomata plants (Bertolino *et al.*, 2019; Caine *et al.*, 2023).

Environmental drivers of leaf traits and iWUE

We observed a strong negative relationship between iWUE and SLA, which is consistent with the findings of previous studies that also reported a strong inverse correlation between these two traits (Dawson *et al.*, 2022; Ge *et al.*, 2022; Petrík *et al.*, 2023, 2024). The negative relationship suggests that plants with lower SLA are typically characterized by thicker or denser leaves and tend to exhibit higher iWUE. The structural features reflect conservative water use strategies, where leaves are structurally adapted to reduce water loss and maintain carbon assimilation under water-limited conditions. On the contrary, higher SLA is generally associated with fast-growing species that prioritize rapid resource acquisition over conservation, often at the expense of water use efficiency.

We found a positive relationship among iWUE and S, SD and maximum g_s, consistent with previous studies. In particular, our results confirm the strong positive relationship between iWUE and SD reported by Bhaskara *et al.* (2022), Al-Salman *et al.* (2023), Caine *et al.* (2023) and Petrík *et al.* (2024). This trend was further experimentally supported experimentally by Franks *et al.* (2015), who demonstrated that *Arabidopsis thaliana* mutants with genetically reduced SD exhibited higher iWUE under controlled conditions. However, numerous other studies have also documented negative relationships between SD and iWUE in crops such as maize (Liu *et al.*, 2015), rice (Pitaloka *et al.*, 2022) or poplars (Jiao *et al.*, 2022; Xia *et al.*, 2024), as well as in several tropical tree species (Pan *et al.*, 2024), often in the context of enhancing drought tolerance under climate change. Studying the photosynthetic performance of *Coffea* spp., Ramalho *et al.* (2013) reported similar patterns: SD decreased, whereas S increased in response to long-term elevated [CO_2]. Our findings suggest that the relationship between SD and iWUE is context-dependent, shaped by environmental conditions, species-specific physiology and growth strategy. A higher SD can facilitate fine regulation of gas exchange, allowing plants to optimize carbon uptake while minimizing water loss, particularly under variable environmental conditions. Although we found a positive relationship between iWUE and SD, evidence from other systems shows that lower SD can also improve water use efficiency. For instance, Petrík *et al.* (2023) reported that reduced SD enhances iWUE by limiting transportational water loss; similar findings were published by Stojnić *et al.* (2019) for 1-year-old *Quercus robur* plants, Bhaskara *et al.* (2022) for *Arabidopsis*, Al-Salman *et al.* (2023) for *Sorghum* and Caine *et al.* (2023) for rice. In our study, the density of the stomata declined over time in concert with a decrease in iWUE, a pattern that may be characteristic of tropical forests of the Congo basin and could increase the vulnerability of *C. canephora* to future climate stress. Additional studies are needed on other tropical species to test this hypothesis.

The positive relationship between g_{cmax} and iWUE suggests that plants with higher anatomical capacity for stomatal opening can maintain efficient water use while achieving greater carbon assimilation, especially under favourable conditions (adequate moisture, moderate temperature). A higher g_{cmax} indicates a greater anatomical capacity for stomatal opening, which facilitates increased gas exchange and high net photosynthesis (A_{net}), which aligns with findings from Ge *et al.* (2022) and Petrík *et al.* (2024). This supports optimized growth and productivity in environments with intermittent water stress. The conceptual isotope model (Scheidegger *et al.*, 2000; Rahman *et al.*, 2020; Vitali *et al.*, 2021; Pu and Lyu, 2023) explains that increasing iWUE can result from enhanced net photosynthesis, reduced stomatal conductance, or a combination of both. The positive g_{cmax}–iWUE relationship seems counterintuitive, as a higher g_{cmax} typically implies increased g_s, which could reduce iWUE by increasing water loss. This suggests that the enhanced photosynthesis rate likely plays a dominant role in driving the observed increase in iWUE, outweighing potential water loss from higher conductance, especially under favourable conditions. However, the exact physiological underlying mechanisms remain unclear, which warrants further research on how stomatal regulation, photosynthetic capacity and environmental factors interact to shape this relationship. Several environmental factors can influence the physiological traits measured in herbarium specimens, including seasonal timing, diurnal variation and microclimatic conditions at the time of collection. Photosynthetic rates (A_{net}) and stomatal conductance (g_s) can vary substantially across the day due to changes in light, temperature and humidity, and can also differ seasonally depending on rainfall and temperature patterns (Miao *et al.*, 2021; Ramalho *et al.*, 2025). Additionally, microclimatic variation among collection sites, such as differences in canopy cover or local soil moisture, may further affect leaf physiology. Because herbarium specimens were collected under a wide range of uncontrolled conditions, with a lack of information regarding land use change, forest density change, site openness, or the exact time of collection during the day, we acknowledge these sources of variation as inherent limitations of using historical data and caution that they may contribute to some of the observed variability in our results. Experimental studies, such as those that manipulate water availability or temperature, could help clarify these dynamics.

Decoupled spatial and temporal responses of functional traits

While the previous section addressed individual trait relationships with iWUE, we now turn to how these traits vary across space and time under environmental gradients. Our analysis revealed a pronounced spatial and temporal variation in the functional leaf traits of *C. canephora* shrubs in the Congo Basin, encompassing sites distributed along an altitudinal gradient from 450 m in the Kasaï and Yangambi regions to 800 m in the Epulu region. Altitude is often associated with climatic variation, and this heterogeneity likely contributes to spatial differentiation in leaf trait expression (Körner, 2007; Mujawamariya *et al.*, 2018; Fyllas *et al.*, 2020).

Morphological traits, including SLA, S and SPS, were more strongly shaped by spatial climatic gradients, especially MAT and latitude. On the contrary, physiological traits such as g_{cmax} and C_{i} exhibited a greater sensitivity to C_{atm} and MAP, suggesting a more dynamic response to transient environmental drivers. This divergence in trait sensitivity supports a pattern of functional decoupling, in line with findings from other ecosystems, in subtropical forests, where Zhu *et al.* (2022) found variation in physiological traits in coordination with structural traits. At the community level, Gao *et al.* (2022) found that in nutrient-rich environments plants may increase SLA to capture more light, while in nutrient-poor conditions they may reduce SLA to enhance leaf longevity, as effects of environmental filtration on community SLA are mainly realized by climatic factors (e.g. temperature and rainfall) and soil nutrients. Wright *et al.* (2004) described similar global trait–climate associations in the leaf economics spectrum. The observed increase in SLA with MAT is consistent with patterns documented across multiple biomes, where plants in warmer environments tend to produce thinner leaves with higher SLA to enhance carbon gain under favourable thermal and light conditions, positively correlated with photosynthetic traits (Wright *et al.*, 2005; Poorter *et al.*, 2009; Li and Prentice, 2024). In particular, SLA in *C. canephora* did not show a significant response to CO_2 or precipitation, indicating that its variation is more closely aligned with temperature-driven gradients than with hydric or atmospheric CO_2 drivers, as also reported in tropical tree species (Martínez-Vilalta *et al.*, 2014; Hatangi *et al.*, 2023).

Latitudinal variation in stomatal and pore size further supports the hypothesis of morphological acclimatization or adaptation to broad-scale climatic gradients. Larger stomata and pores observed at higher latitudes may facilitate CO_2 uptake under conditions of reduced light and cooler temperatures, compensating for potentially lower photosynthetic rates (Woodward and Kelly, 1995; Carins Murphy *et al.*, 2014). These patterns are consistent with previous work showing that stomatal traits covary with temperature and irradiance along latitudinal gradients (Hetherington and Woodward, 2003; Milla, 2023). Although SD was not significantly influenced by any single environmental variable in our study, a marginally negative association with temperature seasonality suggests that climatic stability could regulate stomatal development, as supported by studies in other tropical and temperate systems (Peñuelas *et al.*, 2011).

Physiological traits responded distinctly to environmental variation. We observed a significant decrease in g_{cmax} and an increase in C_{i} with increasing CO_2, indicating that *C. canephora* adjusts stomatal behaviour to minimize water loss while maintaining or improving internal CO_2 concentrations. This response is consistent with global evidence that increased atmospheric CO_2 leads to reduced g_{s} across diverse plant taxa (Franks *et al.*, 2013; Keenan *et al.*, 2013; Lin *et al.*, 2015). Furthermore, the positive effects of both MAT and MAP on C_{i} suggest that leaf physiological functioning is sensitive to both thermal and hydric conditions, an interpretation consistent with previous findings in tropical rainforest trees and lianas (Anderegg *et al.*, 2016; Slot and Winter, 2017).

Substantial differences between marginal and conditional R^2 values in our mixed-effects models highlight the importance of unmeasured variation, including soil properties, nutrient availability and microclimatic factors. Incorporating region and year as random effects helped account for this context-dependent variability, emphasizing the necessity of considering spatiotemporal heterogeneity in ecological trait analysis (Messier *et al.*, 2010; Albert *et al.*, 2011). Overall, our results support a functional divergence in trait–environment relationships: morphological traits appear to reflect long-term adaptation to spatial climatic gradients, while physiological traits exhibit more plastic, short-term responses to atmospheric and hydric changes. This decoupling may allow *C. canephora* to maintain ecological performance in the face of environmental changes (Valladares *et al.*, 2007; Gratani, 2014). However, part of the discrepancy between our findings and some previous knowledge of *Coffea* spp. may stem from methodological differences, including sampling strategies, environmental characterization of sampling sites, or trait measurement protocols. Such sources of variation highlight the importance of harmonized approaches in trait-based studies to distinguish biological signals from methodological artefacts, although these potential biases are particularly difficult to control when utilizing herbarium material. Future studies should aim to disentangle the genetic and plastic contributions to these trait responses through common garden or reciprocal transplant experiments (Nicotra *et al.*, 2010) and expand trait-based analyses to include reproductive and below-ground traits for a more comprehensive understanding of species-level adaptation strategies in both cultivated and wild populations.

Conclusions

Our study provides strong evidence that *C. canephora*, a key understorey species in the Congo Basin, has undergone significant changes in both leaf morphology and physiology over the past century. Through analyses of herbarium specimens collected between 1900 and 2021, we observed long-term trends indicative of potential acclimatization and/or adaptation to increasing atmospheric CO_2 and microclimatic changes. Specifically, increases in SLA, stomatal size and intercellular CO_2 concentration (C_{i}) have been noted. In parallel, decreases in stomatal density, stomatal pore size, maximum diffusive stomatal conductance to CO_2 (g_{cmax}), $\delta^{13}C$ and iWUE, indicating a shift towards more conservative water use strategies, have been found in the Congo Basin.

The coordinated trait changes point to complex physiological adjustments, potentially shaped by both environmental constraints and functional trade-offs. Importantly, the observed decrease in iWUE, despite elevated CO_2, challenges expectations from stomatal optimization theory. Spatial analysis carried out through our study further revealed that morphological traits responded primarily to long-term climatic gradients, while physiological traits were more sensitive to transient drivers such as atmospheric CO_2 and precipitation.

Overall, our findings reveal the remarkable plasticity of *C. canephora*, but also challenge its vulnerability in the context of global change. They underscore the value of integrating temporal, spatial and trait-based approaches to better understand plant adaptation strategies in tropical forest ecosystems. Future research should expand to other understorey species and integrate physiological measurements with demographic and reproductive traits to refine predictions of species resilience under ongoing climate change.

SUPPLEMENTARY DATA

Supplementary data are available at Annals of Botany online and consist of the following: Table S1: data representing the morphological and physiological traits of the 179 *Coffea canephora* herbarium specimens analysed in this article. Table S2: results of LMMs testing the effects of year, region and their interaction on leaf functional traits of *Coffea canephora*. Table S3: results of LMMs testing the effects of region, period and their interaction on foliar traits of *Coffea canephora*. Figure S1: temporal trends in mean annual temperature at the main sampling sites from 1901 to 2024. Figure S2: temporal trends in annual precipitation at the main sampling sites from 1901 to 2024. Figure S3: relationships between leaf morphological traits and iWUE in *Coffea canephora* from forests of the Congo Basin based on recent herbarium samples collected between 2016 and 2022. Table S1 that support the findings of this study is available on Zenodo at https://zenodo.org/records/17176173.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

All authors conceived the ideas and designed the methodology. Y.H., J.D. and A.-S.T. collected the data. Y.H., F.V. and M.B. analysed the data. Y.H., F.V., P.B. and M.B. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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