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RESEARCH ARTICLE

Leaf traits of understory woody species in the Congo Basin forests changed over a 60-year period

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Abstract

Background and aims – While tropical forests play an important role in carbon sequestration, they are assumed to be sensitive to rising temperatures and prolonged drought. Plant functional traits are useful for understanding and predicting the effects of such changes in plant communities. Here, we analyse the variation of leaf traits of understory woody species of the Congo Basin rainforests over a 60-year period using herbaria as tools and we verify if this variation is potentially related to recent climate change.
Material and methods – Leaves of five shrub species were collected in 2019–2022 in Congolese old-growth forests (Yangambi Biosphere Reserve, DR Congo) from different positions on the shrub. These leaves were compared with herbarium specimens collected in the same area before 1960. For both periods, we assessed leaf size, specific leaf area, stomatal size, and stomatal density for all species.
Key results – The variability of the functional traits of the understory woody species are independent of the position of the leaves in the crown. This allows for the use of historic herbarium collections for trait analyses on tropical understory shrubs. The traits of the recently collected leaves were notably different from the traits of herbarium leaves collected in pre-1960: recent leaves were significantly larger, had a higher Specific Leaf Area, a smaller stomata pore length, and, apart from *Coffea canephora*, showed a lower stomatal density.
Conclusion – The difference in traits over time is probably related to the increase in temperature and to atmospheric CO_2 concentration, as the average temperature at Yangambi over the past 60 years has shown an upward trend consistent with global increasing CO_2 levels, while the average annual rainfall has remained unchanged. Our results provide a first insight into the response of forest species to climate change in the Congo Basin forests, and on how the understory species and the ecosystem will react in the long term, when the temperature further increases.

Keywords

climate change, Congo basin, leaf traits, understory woody species

INTRODUCTION

Tropical forests are characterised by their high diversity of woody species (Ter Steege et al. 2013; Kearsley et al. 2017; Rahman et al. 2019) and are well known for the complexity of their vertical structure (Poorter et al. 2006). Furthermore, these forests are huge carbon sinks (Pan et al. 2011), provide numerous ecosystem services, and play an important role in climate change regulation (van der Sleen et al. 2014). Understanding species and phenotypic diversity of tropical forest plants is essential to predict effects of climate change on tropical forest and to

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implement appropriate conservation measures (Kappelle et al. 1999; Mcclean et al. 2005).

Species from tropical forests are sensitive to rising temperatures and prolonged drought conditions, because they are accustomed to low temperature variation (Lewis et al. 2009; Bretfeld et al. 2018; Tng et al. 2018). Climate change has already affected tropical forests during past geological time (Maley 2004), leading to, for example, the appearance of forest refugia between 2500 and 2000 years before the present (Maley 2004; Maley et al. 2018). Climate change effects in tropical forests are expected to manifest through increases in average annual temperatures and a significant decrease in precipitation (Bretfeld et al. 2018). Under the current climate change scenarios, African tropical forests will experience a temperature increase of 3 to 4°C by the end of the 21st century. Moreover, it is confirmed that all tropical forests have already been affected by abrupt warming with an average rate of 0.26 ± 0.05°C per decade since 1970 (Malhi and Wright 2004).

Predicting the effects of climate change on forest ecosystems remains an ecological challenge (Bellard et al. 2012; Soudzilovskaia et al. 2013; Kafuti et al. 2020). The impact of new climate regimes on plant physiology is key to predicting the future distribution of tropical forests (Zelazowski et al. 2011) and its species composition. Such predictions benefit substantially from detailed knowledge on plant functional traits that correlate very well with the ecological performance of plants, and which play an important role in the assembly of plant communities (Niklaus et al. 2017; Gao et al. 2022). As such, they are useful tools to predict possible changes in plant communities and in their functions, in response to climate change (Pérez-Harguindeguy et al. 2013; Soudzilovskaia et al. 2013).

Leaf functional traits are highly plastic and tightly related to environmental conditions, such as the light environment, CO_2-levels, and water and nutrient availability (Wright et al. 2004; Osnas et al. 2013). Specific leaf area (SLA) is an important functional trait, which is strongly related to the ability of plants to capture light (Gao et al. 2022). The SLA is associated with other plant functional traits, leaf gas exchange, and plant growth, and is at the centre of a nexus of covarying traits that together affect the ecology of plant species (Shipley 1995). The density and size of plant stomata are good bio-indicators of local changes in air composition and can be used to assess the effects of climate change on tropical forests (Woodward et al. 2002; Koffi et al. 2014; Tian et al. 2016). Generally, leaves formed during periods characterised by high temperatures are expected to have higher spacing between stomata, thus reducing the stomatal density of the leaves (Woodward et al. 2002; Soudzilovskaia et al. 2013). Furthermore, it has been shown that changes in CO_2 concentrations in the air can trigger changes in the stomatal density of plants. This provides evidence that plants can detect and respond to the effects of ecosystem anthropisation, such as changes in atmospheric composition (Woodward 1987; Beerling and Chaloner 1993; Tian et al. 2016). Since stomatal density is negatively correlated with the increase in CO_2 in the atmosphere (Woodward et al. 2002), a greenhouse gas responsible for global warming, studying its variation over short periods of time would make it possible to understand the plasticity of tropical forest species.

A recent study has shown that understory woody species are more resilient than previously expected to drought and to other environmental changes predicted in tropical forests (Alonso-Rodríguez et al. 2022). However, as most studies in the tropics are focused on canopy trees, little is still known about the tolerance of understory species to changing conditions (Royo and Carson 2006; Tng et al. 2018). Therefore, the main aim of this study is to analyse whether climate change and increased CO_2-levels over the past 60 years may have already impacted leaf traits of shrubs growing in the understory of the Congo Basin rainforests. For five tropical understory species, we analysed the variation in leaf functional traits within individual shrubs or trees, across a period that spans at least 60 years using historic herbarium specimens and leaves collected recently in the Yangambi Biosphere Reserve (DR Congo). Two key questions were formulated. Firstly, do leaf functional traits differ at different positions in the crown of understory woody trees? Since light conditions in the tropical forest understory are much less variable than in the canopy, we expect no significant variation in leaf functional traits within different parts of the tree crown for understory woody trees. Secondly, did leaf functional traits in these understory shrubs change notably over the past 60 years? Since CO_2 levels and mean annual temperature have changed significantly at the global scale over the past 60 years, similar changes could be expected in the Congo Basin and this may have resulted in changes in leaf functional traits.

MATERIAL AND METHODS

Study site

The study was conducted in the Yangambi Biosphere Reserve (YBR), located centrally in the Congo Basin, within the territories of Isangi and Banalia, Tshopo province in the Democratic Republic of Congo (Ebuy et al. 2016; Kearsley et al. 2017). The YBR lies between 00°38' and 01°10'N and 24°16' and 25°08'E, with altitudes varying between 350 and 500 m (Amani 2011). The climate of Yangambi belongs to the Af type according to the Köppen classification (Mohymont and Demarée 2006). The monthly average temperature ranges between 22.4 and 29.3°C, with an annual average of around 25°C (Alongo et al. 2013). In Yangambi, annual rainfall varies from 1500 to 2400 mm, with an average rainfall of 1800 mm. The Yangambi region is dominated by ferralsols, with a clay content varying between 20 and 45%. The soils of Yangambi are reputed to be poor in assimilable mineral matter, due to their high acidity and low retention capacity

Table 1. Species included in this study and the minimum and maximum height of the sampled individuals.

Species	Family	H_{min} (m)	H_{max} (m)
Coffea canephora Pierre ex A.Froehner	Rubiaceae	3	12
Hua gabonii Pierre ex De Wild.	Huaceae	3	12
Scaphopetalum thonneri De Wild. & T.Durand	Malvaceae	4	9
Tabernaemontana penduliflora K.Schum.	Apocynaceae	3	7
Uvariopsis solheidii (De Wild.) Robyns & Ghesq.	Annonaceae	3	8

(Ebuy et al. 2016). They are acidic with a pH between 3 and 4. The vegetation of Yangambi is characterised by a mixture of old-growth and regrowth forests (Tarelkin et al. 2016).

Species selection and sampling

In this study, we used leaves from mature plants, collected fresh and subsequently dried as outlined below, as well as dried leaves from historic herbarium specimens. Fresh leaves were collected from five tropical understory woody species in 2019–2022 (Table 1). These species were selected because they are among the most abundant in the understory of the YBR and belong to different plant families.

Ten individuals per species were selected in old-growth forests in the YBR for leaf sampling. The height of the sampled shrubs varied between 3 and 12 m, as to avoid ontogenetic bias by sampling of young shrubs. From each shrub, three leaves were collected at the top of the crown, three in the middle, and three at the base. Leaves that were diseased or had been browsed by animals were not sampled. All leaves were pressed in a herbarium press and dried in an oven at a temperature of 60°C for 3 days and subsequently air dried for at least four more weeks, in order to make them comparable to the historic herbarium specimens.

For each species, 20 herbarium specimens originally collected in the same section of the Yangambi Biosphere Reserve before 1960 (hereafter named pre-1960) were selected from the Yangambi (YBI) and the Meise Botanic Garden (BR) herbaria. From each herbarium specimen, a leaf was sampled while taking care to not destroy the voucher. All leaves used in this study were originally collected in the Yangambi region according to their labels.

Leaf trait measurements

Leaf trait analyses were performed on 459 newly collected leaves (9 leaves per individual, 10 or 11 shrubs per species, and 5 species) and on 135 old herbarium leaves (1 leaf per specimen, 5 species and 20 leaves for *C. canephora* and *S. thonneri*, 27 for *H. gabonii*, 42 for *T. penduliflora* and 26 for *U. solheidii*). The dry mass of stalkless leaves was determined using a precision balance (precision: 0.0001 g). The upper surface of the leaves was scanned at a resolution of 300 dpi using an EPSON 10000 XL scanner. The surface area of the leaves was calculated using ImageJ v.1.52a (US National Institutes of Health; https://imagej.nih.gov/ij/). The specific leaf area (SLA), which corresponds here to the surface area of one side of the dried leaf divided by its dry mass, was calculated.

On the abaxial surface, a thin layer of colourless nail varnish was applied on both sides of the main vein and dried overnight. The nail varnish was then meticulously detached using transparent tape and glued on a microscopy slide. Two impressions were made for each extant leaf. On each herbarium leaf, two prints at the top, two prints in the middle, and two prints at the base of the leaf were made. Three photos per impression were taken per leaf print at a ×1,000 lens magnification using a digital microscope (VH-5000, Ver 1.5.1.1, Keyence Corporation). The stomata were counted on the photos using ImageJ v.1.51n in a grid of 40,000 μm² surface area. The stomatal density (SD) of each species was calculated and expressed per mm². On each of the photos taken, the length and width of one single representative and clear stoma was measured. At the same time, the length and width of the same stoma pore was measured using the ObjectJ plugin in ImageJ (Pérez-Harguindeguy et al. 2013). The stomatal size was calculated by multiplying stomata length by stomata width (Franks and Beerling 2009). These data have been deposited in Zenodo and are available at https://doi.org/10.5281/zenodo.8130615.

Yangambi climatological data

Climatological data consisting of average monthly rainfall, minimum and maximum temperatures, and monthly averages, covering the period 1961–2021, obtained directly from the INERA Yangambi climatology station (KP5), located at 00°49'12" N, 24°27'18" E (see Yakusu et al. 2022 and supplementary material 2 for details).

Changes in mean annual temperature and rainfall in Yangambi over the past 60 years were highlighted by analysing temperature and rainfall data. The identification of any trend in the time series was done using the Mann-Kendall trend test, performed using the Mann-Kendall function of the package Kendall v.2.2.1 in R v.4.2.1. It was applied separately to the precipitation and temperature series.

In Yangambi, the average annual temperature recorded for the period 1961–2021 was 24.98°C. The annual minimum temperature of 18.93°C was recorded in 1985,

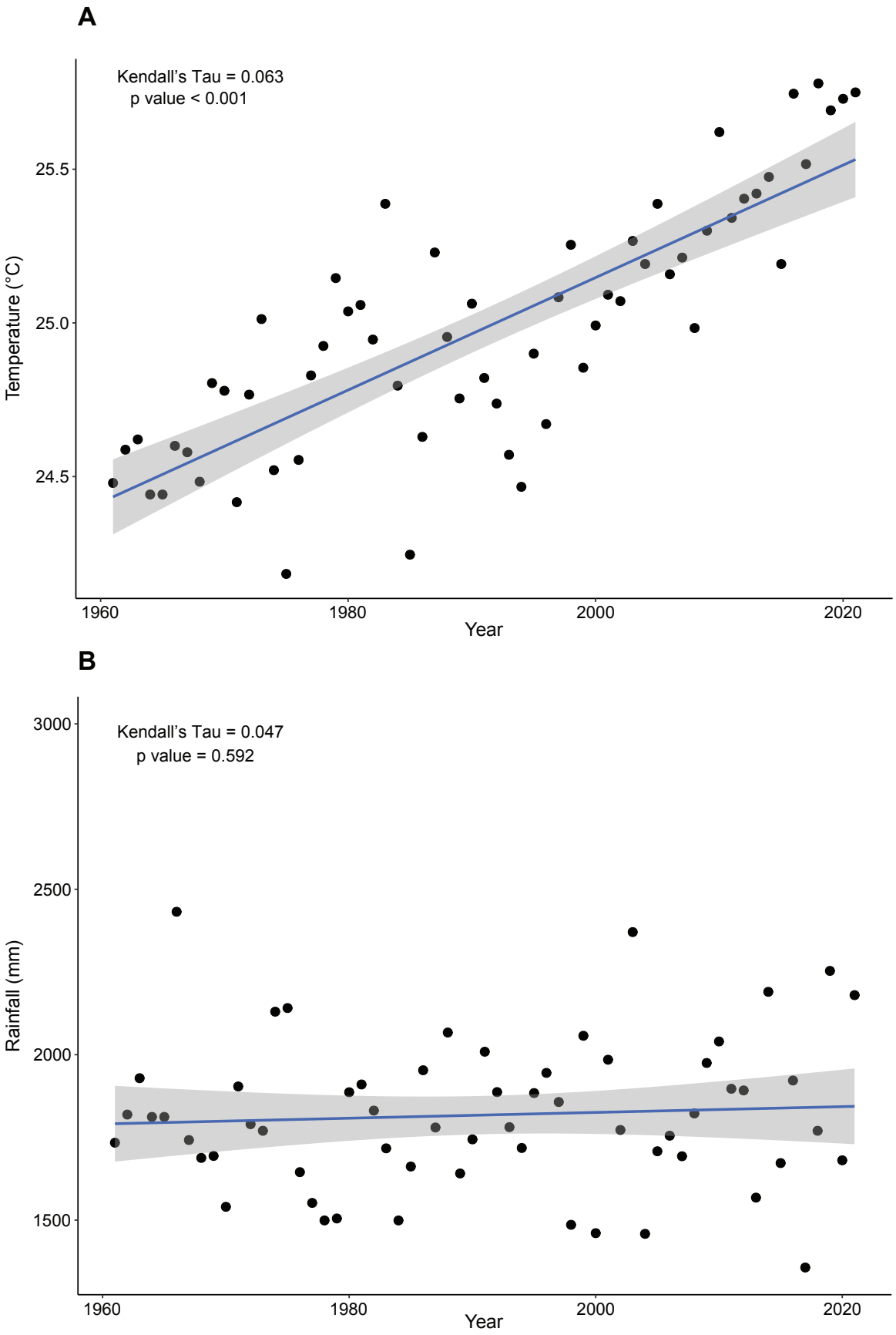


Figure 1. Annual mean temperature (**A**) and rainfall (**B**) recorded at Yangambi from 1961 to 2021 (raw data received from INERA, Yangambi, see Yakusu et al. 2022 for details).

Table 2. Results of the ANOVA on linear mixed-effects models comparing leaf functional traits at three different positions (top, middle, bottom) of five tropical understory species.

Leaf traits	Position effect		Species effect		Position × Species	
	F value	p value	F value	p value	F value	p value
Leaf area	0.55	0.75	43	< 0.001	9.05	0.34
Leaf dry mass	1.23	0.54	29.69	< 0.001	10.72	0.22
Specific leaf area	0.51	0.77	13.54	< 0.001	10.72	0.22
Stomata length	0.61	0.73	329.23	< 0.001	4.71	0.79
Stomata width	0.71	0.7	66.55	< 0.001	2.95	0.94
Pore length	0.67	0.71	123.34	< 0.001	2.04	0.98
Pore width	0.71	0.69	20.98	< 0.001	5.48	0.71
Stomatal density	2.71	0.25	244.93	< 0.001	8.63	0.37

while the annual maximum temperature of 30.8°C was recorded in 2016. There was an upward trend in the temperature time series for this period (Kendall's Tau = 0.63, p value < 0.001; Fig. 1A). An increase of 1°C was recorded at Yangambi for the entire period: the mean annual temperature was 25.75°C in 2021, compared to 24.48°C in 1961.

The minimum annual rainfall recorded at Yangambi was about 1418 mm in 2017, while the maximum annual rainfall was about 2432 mm in 1966, with an annual mean of about 1817 mm for the entire period. No trend was found in the rainfall distribution series at Yangambi during the period 1961–2021 (Kendall's Tau = 0.047, p value = 0.59; Fig. 1B).

Data analysis

All data were analysed using R v.4.2.1. The normality of the data and the distribution of the residuals was checked using a Shapiro-Wilk test for all traits before other statistical test were performed A logarithmic transformation was performed on all trait data to meet normality requirements. In order to quantify the sources of variability in the traits depending on the position of the leaf in the canopy, we fitted linear mixed-effects models using the lmer function of the package lme4 v.1.1-31 (Bates et al. 2015), with leaf position and species considered as fixed effect and individuals as random effect. Afterwards, the results of all models were evaluated using the Anova function of the package car v.3.1-2 (Fox and Weisberg 2018). To compare the historic herbarium samples to the extant leaves, and to understand if there was variability in the traits depending on the two periods considered in this research, we fitted the linear models. As we do not know the position within the shrub where the material was sampled for the historic herbarium samples, we first tested the influence of the position within the crown on the traits. As there was no effect of leaf position, we compared the historic herbarium samples with recent samples and excluded a possible sampling bias.

RESULTS

Variability in leaf traits according to leaf position in the crown

There were no significant differences in leaf traits located at different levels in the crown, for any of the traits measured, nor a significant interaction between position and species (Table 2). We did, however, find significant differences between species for all leaf traits.

The largest leaves in terms of area and dry mass were observed in *Coffea canephora* (Fig. 2A, B), while *Hua gabonii* had the smallest leaves (Fig. 2A). The highest SLA was observed in *Tabernaemontana penduliflora* (212.28 ± 114.44 $cm^2.g^{-1}$) and the lowest in *Uvariopsis solheidii* (144.94 ± 51.88 $cm^2.g^{-1}$) (Fig. 2C).

There was no significant difference in stomatal size measured for leaves collected at different positions in the crown (Table 2). Indeed, leaf stomata had lengths around 23.43 ± 6.38 µm regardless of the position of the leaves in the crown (Fig. 2D), whereas their widths oscillated around 15.87 ± 4.82 µm on average (Fig. 2E). Overall, the largest stomata were measured on specimens of *U. solheidii* and the smallest on specimens of *H. gabonii*. The stomatal pore length of all species studied was distributed around 15.54 ± 4.3 µm (Fig. 2F). Similarly, the stomatal pore width of all species studied was distributed around the mean value 7.17 ± 2.26 µm (Fig. 2G).

Hua gabonii had a considerably higher stomatal density compared to other species, with a mean of 695 ± 40 $stomata.mm^{-2}$. The species *T. penduliflora* had the lowest stomatal density with a mean of 155 ± 65 $stomata.mm^{-2}$ (Fig. 2I).

Changes in leaf traits between pre-1960 and 2019–2022

Several leaf traits of understory woody species changed significantly between the periods pre-1960 and 2019–2022 in the Yangambi Biosphere Reserve (Table 3).

The surface area of leaves collected in 2019–2022 (133.62 ± 60 cm^2) was significantly larger than the one of specimens collected in pre-1960 (104.18 ± 55 cm^2; Table 3; Fig. 3A). The leaf mass was not different between the two time periods, but there was a significant interaction effect with species (Table 3). The leaf mass of *H. gabonii* was higher in 2019–2022 than in pre-1960, while the leaf mass was lower for *C. canephora*, *S. thonneri*, and *U. solheidii* collected in 2019–2022. Leaves collected in 2019–2022 showed a significantly larger specific leaf area (182.19 ± 79.1 $cm^2.g^{-1}$) when compared to those collected in pre-1960 (137.21 ± 28.51 $cm^2.g^{-1}$; Table 3; Fig. 3C).

The length and width of stomata of the five studied species in the YBR understory did not change over the past 60 years, nor was there a significant interaction effect (Table 3; Fig. 3D, E). In contrast, pore length did change over this time period (Table 3; Fig. 3F). Leaves collected in 2019–2022 had shorter pores (15.54 ± 4.3 µm) than leaves from pre-1960 (16.88 ± 6.49 µm). In addition, there was an interaction effect with a more pronounced decrease in pore length for *S. thonneri*, *T. penduliflora*, and *U. solheidii*. Pore width showed no significant difference between the two time periods (Table 3; Fig. 3E).

There was a significant interaction between collecting period and species for stomatal density (Table 3). Four out of five woody species studied showed a decrease in the number of stomata per unit area during the 2019–2022 period (320 ± 203 stomata.mm^{-2}) compared to pre-1960 (339 ± 239 stomata.mm^{-2}; Fig. 3I). Only *C. canephora* showed an increase in stomatal density from 244 ± 40 stomata.mm^{-2} in pre-1960 to 298 ± 60 stomata.mm^{-2} in 2019–2022.

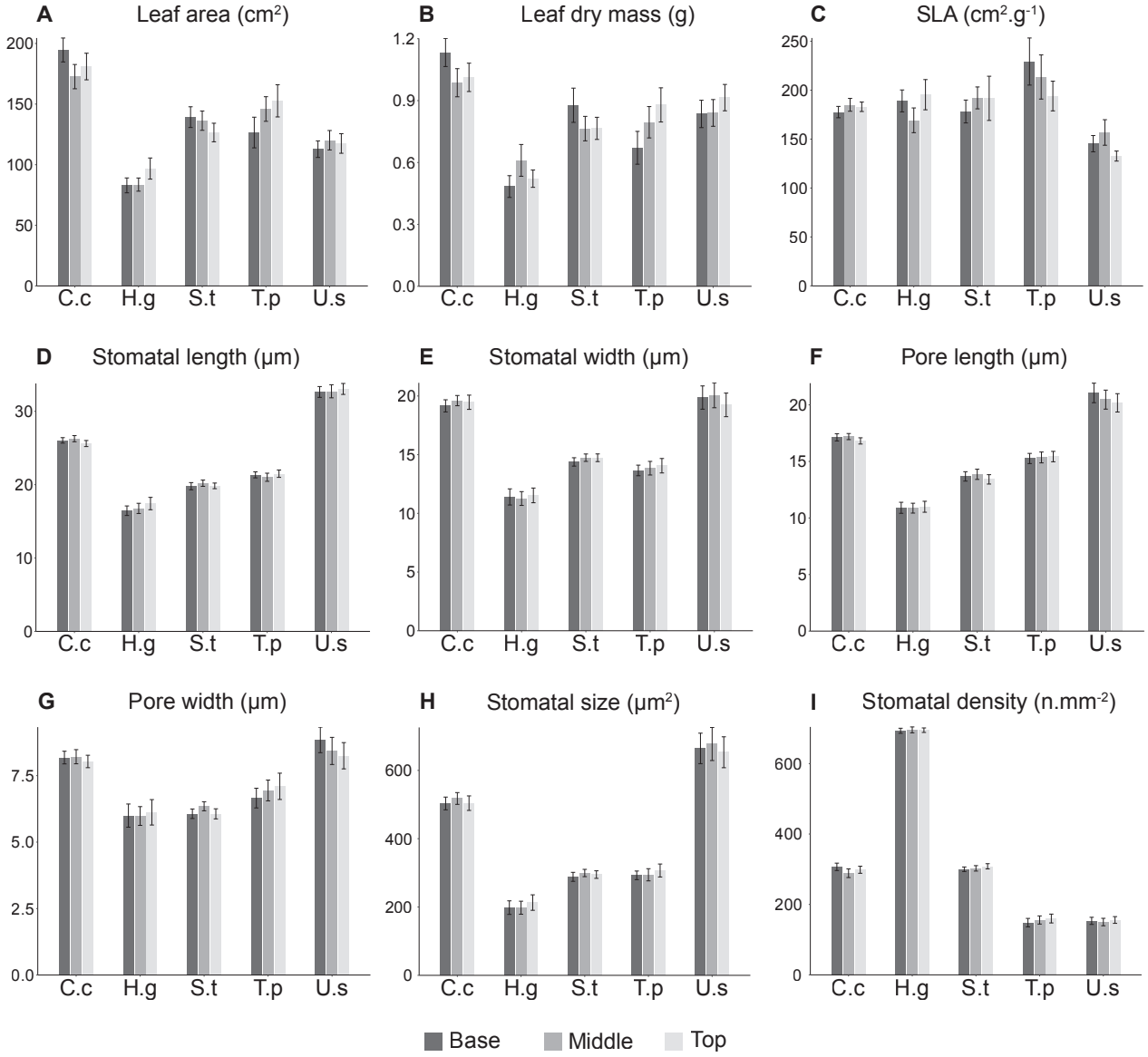


Figure 2. Variation in leaf traits as a function of position at different crown levels of the shrubs: the means of leaf area, dry mass, and specific leaf area of the different species studied. The thick bars are means, while the small bars above are standard error (SE). C.c = *Coffea canephora*, H.g = *Hua gabonii*, S.t = *Scaphopetalum thonneri*, T.p = *Tabernaemontana penduliflora*, U.s = *Uvariopsis solheidii*.

Table 3. Results of the ANOVA on linear models comparing leaf functional traits at two different periods (pre-1960 and 2019–2022) of five tropical understory species.

Leaf traits	Period effect		Species effect		Period × Species	
	F value	p value	F value	p value	F value	p value
Leaf area	31.13	< 0.001	89.57	< 0.001	3.08	< 0.05
Leaf dry mass	3.57	0.5	99.76	< 0.001	3.27	< 0.01
Specific leaf area	93.84	< 0.001	22.23	< 0.001	0.93	0.44
Stomata length	0.02	0.89	409.53	< 0.001	2.15	0.07
Stomata width	0.02	0.89	148.89	< 0.001	1.77	0.13
Pore length	5.63	0.02	309.82	< 0.001	4.63	< 0.001
Pore width	3.25	0.07	51.65	< 0.001	0.83	0.51
Stomatal density	1.18	0.28	7.05	< 0.001	0.05	< 0.001

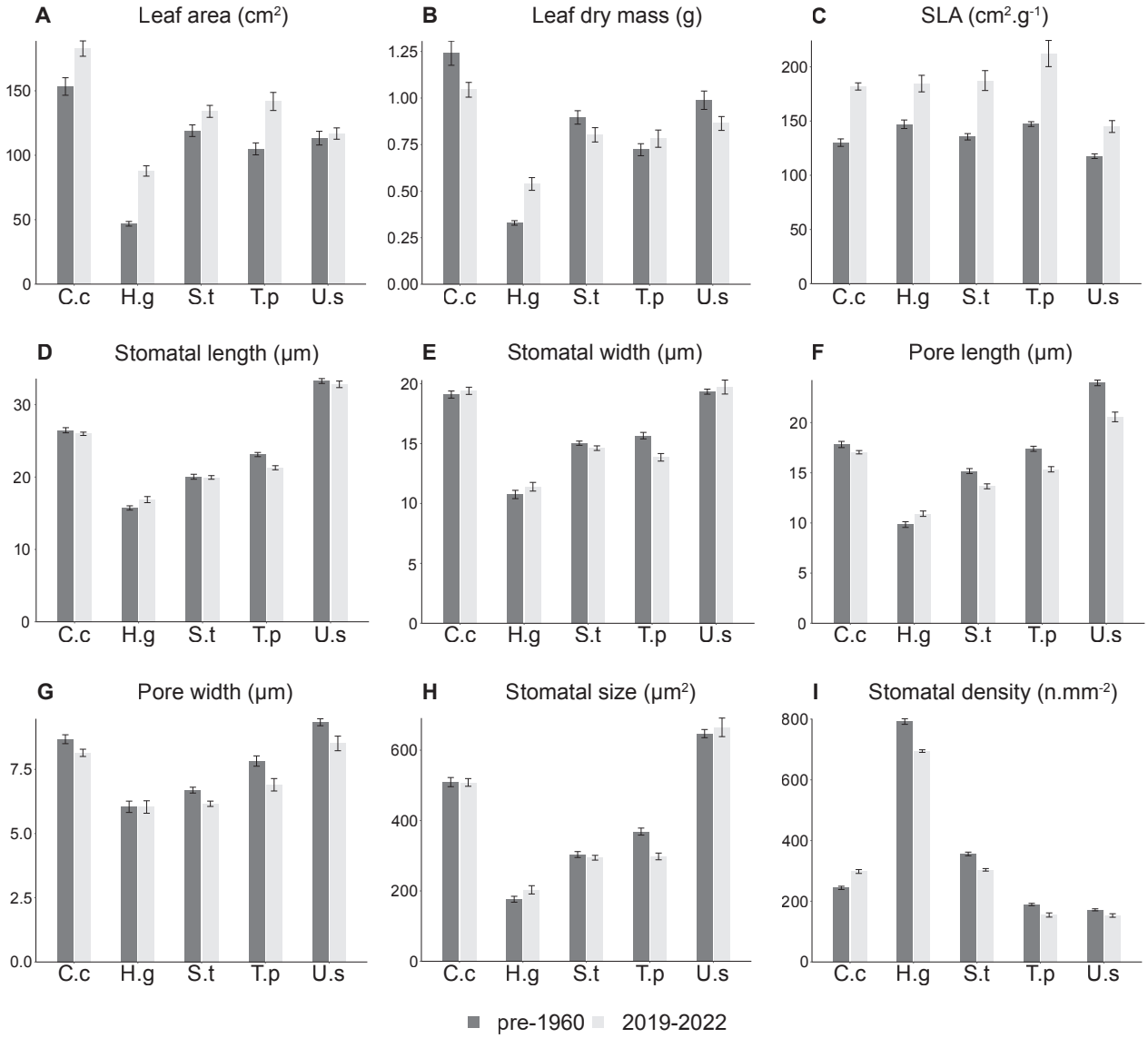


Figure 3. Variation of leaf traits of understory woody species between pre-1960 and 2019–2022. The bars represent average values, while the error bars are standard errors (SE). C.c = *Coffea canephora*, H.g = *Hua gabonii*, S.t = *Scaphopetalum thonneri*, T.p = *Tabernaemontana penduliflora*, U.s = *Uvariopsis solheidii*.

DISCUSSION

As shown in this study, comparing leaves of historic herbarium specimens with the leaves collected for leaf traits in the understory of tropical forests provides a high added value to existing methods, such as short-term experiments or distribution modelling, for studying climate change effects on plants. Our data shows that the leaf position in the tree crown (base, middle, top) has no influence on the trait values of woody species in the forest understory. On the other hand, we did observe changes in leaf characteristics, such as SLA and SD, over at least the past 60 years, that may be related to environmental changes.

Range of trait values

The stomatal density values found in our study are consistent with those found by other authors in other tropical regions (Hultine and Marshall 2000; Hetherington and Woodward 2003). In Côte d'Ivoire, Djinet et al. (2016) recorded 50 stomata.mm^{-2} on leaves of adult *Elaeis guineensis* Jacq., while Camargo and Marenco (2011), studied 35 tropical forest tree species, reported that stomatal density ranged between 110 stomata.mm^{-2}. in *Neea altissima* Poepp. & Endl. and 846 stomata.mm^{-2} in *Qualea acuminata* Spruce ex Warm. Overall, we found a negative relation between stomatal density and stomatal size across the five species, which was more pronounced in recently collected leaves. Such a negative correlation between density and size of stomata has been observed in many other studies (Franks et al. 2009; Fanourakis et al. 2015; De Boer et al. 2016; Tian et al. 2016; Kafuti et al. 2020). The Specific Leaf Area in the species studied ranged from 42.74 cm^2.g^{-1} for *Hua gabonii* to 778.45 cm^2.g^{-1} for *Scaphopetalum thonneri*. These values are similar to those reported in a study on Brazilian forests (Hoffmann et al. 2005). Generally, a low SLA reflects the nutrient poverty of the environments (Tian et al. 2016; Gao et al. 2022).

Leaf position in the tree crown and leaf traits

The uniformity of leaf traits in different positions of the crown can be explained by the fact that all leaves of the species from the understory are exposed to rather uniform light conditions. This is very much unlike leaves from trees in the canopy, whose degree of exposure to light differs depending on whether the leaves are found at the top, middle, or base of the crown (Bauters et al. 2020). One of the main issues with using herbarium specimens from canopy trees to study climate change effects is the fact that it is very often unknown which position in the crown, and therefore sun exposure, the herbarium leaves had at the moment of collecting (e.g. Bauters et al. 2020). Given that light affects stomatal initiation (Royer 2001; Kouwenberg et al. 2007) as well as other functional traits (Poorter et al. 2006), light exposure should be accounted for in studies of leaf traits of trees.

Very high chlorophyll levels accompanied by high photosynthetic capacity have been reported for leaves fully exposed to the sun compared to those in the forest canopy that are not sun exposed (Hollinger 1989). The results found in this study are similar to those found for *Ficus benjamina* L. in Côte d'Ivoire, where the Specific Leaf Area and stomatal density did not vary with the height of leaf removal (Koffi et al. 2014). In contrast, a decrease in leaf dry mass from the top to the base of the tree crown was observed in canopy trees in tropical deciduous forests (Ellsworth and Reich 1993). In evergreen forest species, empirical data have shown an increase in leaf dry matter concentration from the base to the crown of canopy trees (Lewandowska and Jarvis 1977; Hollinger 1989). The photosynthetic capacity of the leaves, which is positively correlated with the nitrogen concentration of the leaves (Reich et al. 1995), is in the opposite direction: it is greater at the top than at the base in the forest canopy (Ellsworth and Reich 1993).

Although physiological and biochemical traits were not measured in this study, it is possible they also vary little with leaf position in the crown of trees in the understory. However, given the strong correlation between specific leaf area and photosynthetic capacity (Tian et al. 2016), it would be interesting to check whether there is any variability in physiological (e.g. stomatal conductance) and biochemical traits (e.g. nitrogen and phosphorus concentration) in understory leaves depending on their position in the crown. We realise that other sources of variation in leaf traits, such as ontogenic stage, season, soil nutrient content, and water content (Fortunel et al. 2020; Schmitt et al. 2022) were not accounted for in this study. However, we did make a maximal effort to reduce this variation by sampling individuals in similar conditions in the tropical forest understory and we do not expect that these sources of variation have a major influence on the current conclusions.

In general, our results confirm that studying effects of recent climate change events on tropical forest understory species using herbarium specimens according to modern and standardised protocols (Pérez-Harguindeguy et al. 2013) is more reliable when using tropical forest understory species instead of canopy trees (Peñuelas and Matamala 1990).

Variation in leaf traits of woody species before 1960 and after 2019

In this study, the main objective was to analyse the variation in leaf traits of woody species in the understory of the Yangambi Biosphere Reserve and to check whether there was a potential link between this variation and climate change. Our data showed several changes over time in foliar traits of five species in the understory of the YBR. These changes are potentially linked to climatic changes that have occurred over the past decades.

The main climatic change that has taken place in the Congo Basin is an increase in the temperature, due to

globall increased CO_2 levels, while the annual rainfall has remained constant. For the period 1960–1992 a temperature increase of 1.6°C has been reported throughout the Democratic Republic of Congo (Kazadi and Fukunyama 1996). Recordings from the meteorological institute at INERA Yangambi showed a rising temperature trend between 1961 and 2021 (Fig. 1A). This result is consistent with that of Likoko et al. (2019), who observed that annual mean temperatures increased from 25.23°C to 25.78°C for the two previous decades (2000–2010 and 2010–2018), compared to the mean temperature recorded in Yangambi (Alongo et al. 2013). Furthermore, it corroborates the findings of Kazadi and Fukunyama (1996) who observed an increase in temperature throughout the Congo Basin, including the Yangambi region. In contrast to Kazadi and Fukunyama (1996) who also observed a considerable decrease in rainfall throughout the Congo Basin, the average rainfall remained stable in the Yangambi region between 1960 and 2021, although changes in rainfall seasonality cannot be excluded.

For tropical forest understory species from the Congo Basin, the stomatal density decreased significantly over the past 60 years for four out five species. This is consistent with the observations of Bauters et al. (2020) who analysed herbarium specimens of canopy trees in the Congo Basin. However, no significant change in stomatal density was observed in herbarium specimens collected over one century of two northern Amazonian tree species (Bonal et al. 2011). A decrease in stomatal density, as we found in our study, has been attributed to the response of plants to environmental conditions such as a temperature increase and the increase of air CO_2 concentration (Woodward 1986; Koffi et al. 2014). A decrease in stomatal density, which is tightly related to stomatal conductance, can be considered a key response to increasing CO_2 levels, potentially resulting in higher intrinsic water use efficiency (iWUE) (Bonal et al. 2011). However, Bauters et al. (2020) found a decrease in iWUE over the past 60+ years in the Congo Basin, despite decreasing stomatal density. Isotope measurements, providing insight in leaf physiological changes, would be very interesting to determine whether such a decrease in iWUE is also found in Congo Basin tropical forestunderstory species. Other factors besides CO_2 levels may also explain changes in stomatal density. An effect of increasing temperature on the decrease in stomatal density, for example, has been experimentally demonstrated (Beerling and Chaloner 1993; Hovenden 2001). An increase in stomatal density with a spatial temperature decrease at higher altitudes has been observed for *Quercus kelloggii* Newb. and *Nothofagus solandri* (Hook.f.) Oerst. species in New Zealand (Kouwenberg et al. 2007). In other studies, it was also clearly demonstrated that stomatal density was positively correlated with altitude (Woodward 1986; Hovenden 2001; Woodward et al. 2002), although there are some exceptions in terms of species and regions (Hultine and Marshall 2000).

The fact that recently collected leaves have higher SLA values may indicate that tropical forests have been enriched with nutrients, and especially atmospheric CO_2, leading to an overall gain in biomass (Lewis et al. 2009; Hubau et al. 2019), as a result of the increased photosynthetic capacity of the leaves. However, in other tropical regions, no increase in plant growth was observed while water use efficiency increased with atmospheric CO_2 enrichment for both understory and canopy species (van der Sleen et al. 2014; but see Bauters et al. 2020). Bonal et al. (2011) did not observe changes in LMA with changing CO_2 levels in two neotropical tree species, but they sampled material over a much wider geographical area leading to potential confounding effects of soil and climate. Generally, it is known that larger leaves have a higher light absorption capacity as well as a higher photosynthetic capacity (Tian et al. 2016). Variation in leaf area was observed along an altitudinal gradient in tropical forests for one bamboo species with a decrease in values with increasing altitude (Guo et al. 2018). The same trend has been observed in New Zealand (Kouwenberg et al. 2007). This spatial trend of increasing leaf size with increasing temperature at lower altitudes is in line with the temporal trend we found as the average temperature in the Yangambi region had risen by 1°C between 1961 and 2021. However, further studies are required to disentangle to which extent the different environmental factors that have changed over the past 60 years have contributed to changes in SLA in our study area.

The results of this study show that these understory species of the YBR may have responded to the changed temperature conditions in the Yangambi region. Although rainfall remained stable in Yangambi, an increased temperature may have resulted in higher evaporation and drier soil conditions. Such drier conditions may also explain the decrease in stomatal density, as a lower stomatal density is generally observed in plants that are drought resistant (Franks et al. 2009; Bertolino et al. 2019).

CONCLUSION

Through this study, we have shown that the leaf position in the tree crown has no effect on leaf traits for woody species in the understory of the Yangambi Biosphere Reserve. This finding shows that herbaria are reliable sources of study material for leaf trait analysis of undergrowth species and that new leaf samples can be studied without considering the vertical stratification of the crown. This would save time as well as human, material, and financial resources.

Furthermore, we have shown that the understory species of the Yangambi Biosphere Reserve may have already modified some of their leaf traits in response to the climatic variations recorded in the region between the periods pre-1960 and 2019–2022. They have developed larger leaves but with fewer stomata than historical specimens. This could be a response to environmental

variation and these changes are likely to continue in the coming years as temperature and atmospheric CO_2 are expected to increase further.

Having sampled only a small number of the numerous species in the understory of the Congo Basin forests, a more extensive study to measure leaf traits is needed for other understory woody species and in more locations in order to build a solid database that can be used to draw more general conclusions about the future of tropical forests.

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SUPPLEMENTARY MATERIALS

Supplementary material 1
Data associated with leaves collected before 1961, and with leaves collected in the period 2019–2021.
Link: https://doi.org/10.5091/plecevo.104593.suppl1

Supplementary material 2
Monthly average temperature and annual rainfall at the INERA Yangambi climatology station (DR Congo) between 1961 and 2021. Source: Yakusu et al. (2022).
Link: https://doi.org/10.5091/plecevo.104593.suppl2