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Functional resilience of wild *Coffea canephora* to climate change in the Congo Basin

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Cover photo: A *Coffea canephora* plant growing in a Congo Basin forest (Domaine de chasse de Rubi-Télé). © Yves Hatangi.

Coffee is not cultivated solely out of necessity, but emerges from an interaction between forest ecosystems, temporal dynamics, and human agency.

This thesis is dedicated to

my father, Hatangimbabazi Claudien, and my mother, Munyabarenzi Jeannette

my wife, Flora Mitengezo Hatangi, and our daughter, Lise Hatangi

my brothers and sisters of the Hatangi family: Maombi, Bora, Ernest, Patrick, Thierry, Bienvenue, Bruce, Aline, Bertin, Rosine, Paul, and Benjamin

my extended family: uncles, aunts, and cousins

all coffee enthusiasts and forest stewards.

Abstract

Background – Understanding plant responses to environmental change is essential for both tropical forest conservation and agricultural resilience. In the Congo Basin, climate change, through rising temperatures, altered rainfall, and increased vapor pressure deficits, may pose growing challenges for understory species whose physiological limits are poorly documented. *Coffea canephora*, native to Central African forests and a key species for global coffee production, is increasingly exposed to these pressures. Despite its ecological and genetic importance for Robusta coffee breeding, its long-term functional responses and adaptive capacity remain poorly understood. This thesis addresses these gaps by integrating research on historical herbarium specimens, recent field data, and altitudinal gradient experiments to assess variation in functional and physiological traits of *C. canephora* across time, space, and environmental conditions.

Methods – The study followed an integrated research approach. First, long-term phenotypic changes were quantified by comparing herbarium specimens collected before 1960 with those collected recently (2019-2022) in the Yangambi Biosphere Reserve. Leaf functional traits were measured using standardized protocols. Second, leaf functional traits, carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotopes, and intrinsic water use efficiency (iWUE) of *C. canephora* were analyzed using herbarium specimens (old and recent) collected throughout the Democratic Republic of Congo (DRC) from 1900 to 2022. Third, multi-location trial was set up along an altitudinal gradient with three genetically distinct varieties of *C. canephora*. The plants were grown at three altitudes (450 to 1,700 m a.s.l.) representing contrasting environments. Growth and morphological traits were monitored for one year. Leaf functional traits were measured at the end of the trial.

Results – Comparisons between historical and recent samples from Yangambi revealed significant long-term shifts in both leaf morphology and stomatal traits. Recent specimens exhibited larger leaves and higher specific leaf area (SLA), indicating lower leaf construction costs consistent with adaptation to modern climatic conditions characterized by elevated temperatures and potentially higher atmospheric evaporative demand. Stomatal pore length decreased in recent samples, suggesting a compensatory adjustment in gas-exchange regulation under warmer and drier conditions. These patterns were robust across understory species, including *C. canephora*, and indicate that understory plants have already undergone phenotypic adjustments over the last century.

The more extensive analysis of old and recent specimens of *Coffea canephora* confirmed substantial spatial and temporal variation in functional traits and iWUE of *C. canephora*. Stomatal density, guard cell dimensions, and maximum diffusive conductance differed significantly across regions, reflecting diverse local climates. Foliar $\delta^{13}C$ and intrinsic water-use efficiency also varied widely, suggesting large differences in long-term carbon acquisition and water-use strategies among populations. Trait correlations revealed consistent trade-offs between carbon assimilation and water conservation, highlighting coordinated ecological responses to environmental gradients. These findings demonstrate that *C. canephora* populations exhibit marked functional differentiation shaped by climatic and geographic variation.

In the multi-location trial, altitude exerted a strong influence on both morphological and physiological traits. Final plant height, number of leaves, and distance between nodes decreased with increasing altitude, reflecting constraints imposed by cooler temperatures on elongation growth. In contrast, plant stem diameter, leaf area, and aboveground biomass (AGB) were higher at the high-altitude site (1,700 m), indicating a shift toward thicker stems and larger leaves under high-altitude conditions. Relative growth rate in leaf area (RGRA) increased with altitude, demonstrating enhanced carbon gain efficiency in cooler environments. Stomatal density declined at higher elevations, while pore length increased, suggesting compensatory regulatory changes in gas exchange. Substantial phenotypic variation among genetic varieties confirmed the presence of meaningful intra-specific diversity that can support future breeding efforts.

Conclusion –This thesis demonstrates that *C. canephora* exhibits strong phenotypic responses to environmental variation and that considerable phenotypic changes have occurred over the past century. Historical herbarium analyses reveal significant trait shifts consistent with a response to changing climate conditions throughout the twentieth century. Contemporary spatial analyses highlight extensive functional differentiation across the species' native range, reflecting both environmental heterogeneity and population-level trait variability. Findings from the multi-location trial confirm pronounced short-term plasticity in growth, leaf morphology, and stomatal regulation along an altitudinal gradient, with distinct genotype origin-specific responses. These findings have direct implications for the conservation of wild coffee genetic resources and for the development of climate-resilient coffee production systems in Central Africa. Safeguarding the species' genetic and ecological diversity will be essential to ensure its persistence in the face of ongoing climate change and to support future breeding efforts aimed at securing sustainable coffee production.

Résumé

Contexte – Comprendre les réponses des plantes aux changements environnementaux est essentiel pour la conservation des forêts tropicales et la résilience agricole. Dans le bassin du Congo, le changement climatique, marqué par la hausse des températures, la modification des régimes de précipitations et l'accroissement du déficit de pression de vapeur, poserait des défis croissants aux espèces du sous-bois des forêts tropicales, dont les limites physiologiques sont encore mal documentées. *Coffea canephora*, originaire des forêts d'Afrique centrale et espèce majeure pour la production du café à l'échelle mondiale, est de plus en plus exposée à ces pressions. Malgré son importance écologique et génétique pour l'amélioration du café Robusta, ses réponses fonctionnelles à long terme et sa capacité d'adaptation restent insuffisamment comprises. Cette thèse vise à combler ces lacunes en intégrant des recherches sur des spécimens d'herbiers historiques, des données récentes de terrain et des expériences le long d'un gradient altitudinal afin d'évaluer la variation des traits fonctionnels et physiologiques de *C. canephora* à travers le temps, l'espace et les conditions environnementales.

Méthodes – L'étude a suivi un dispositif de recherche intégré. Premièrement, les changements phénotypiques à long terme ont été quantifiés en comparant des spécimens d'herbiers collectés avant 1960 avec ceux collectés récentes (2019-2022) dans la Réserve de biosphère de Yangambi. Les traits foliaires ont été mesurés suivant des protocoles standardisés. Deuxièmement, les traits fonctionnels, les isotopes de carbone ($\delta^{13}C$) et d'oxygène ($\delta^{18}O$), et l'efficacité intrinsèque de l'utilisation de l'eau (iWUE) de *C. canephora* ont été analysés ; en utilisant des spécimens d'herbiers (anciens et récents) collectés à travers la République démocratique du Congo (RDC). Les relations entre traits et environnement ont été examinées à l'aide de modèles linéaires à effets mixtes. Troisièmement, des essais multilocaux ont été installés le long d'un gradient altitudinal avec trois variétés génétiquement distinctes de *C. canephora*. Les plantes ont été cultivées à trois altitudes (450 à 1 700 m) représentant des environnements contrastés. Les traits de croissance et morphologiques ont été suivis pendant un an. Les traits fonctionnels des feuilles ont été mesurés à la fin de l'essai.

Résultats – Les comparaisons entre les échantillons historiques et récents de Yangambi ont révélé des changements significatifs à long terme dans la morphologie des feuilles et les traits stomatiques. Les spécimens récents présentaient des feuilles plus grandes et une surface foliaire spécifique (SLA) plus élevée, indiquant des coûts de construction foliaire plus faibles, cohérents avec une adaptation aux conditions

climatiques modernes caractérisées par des températures élevées et une demande évaporative atmosphérique potentiellement plus forte. La longueur de l'ouverture stomatique a diminué dans les échantillons récents, suggérant un ajustement compensatoire dans la régulation des échanges gazeux sous des conditions plus chaudes et plus sèches. Ces tendances étaient robustes à l'échelle de l'espèce, y compris pour *C. canephora*, et indiquent que les plantes de sous-bois des forêts tropicales ont déjà subi des ajustements phénotypiques au cours du siècle dernier.

L'analyse plus large des anciens et récents herbiers de *Coffea canephora* a confirmé une variation spatiale et temporelle substantielle des traits fonctionnels et de l'iWUE de *C. canephora*. La densité stomatique, les dimensions des cellules de garde et la conductance diffusive maximale différaient significativement selon les régions, reflétant la diversité des climats locaux. Les valeurs foliaires de $\delta^{13}C$ et iWUE variaient également largement, suggérant de larges différences dans les stratégies d'acquisition de carbone et d'utilisation de l'eau à long terme entre les populations. Les corrélations des traits ont révélé des compromis cohérents entre l'assimilation du carbone et la conservation de l'eau, mettant en évidence des réponses écologiques coordonnées aux gradients environnementaux. Ces résultats montrent que les populations de *C. canephora* présentent une différenciation fonctionnelle marquée, façonnée par la variation climatique et géographique.

Dans les essais multilocaux, l'altitude a exercé une influence importante sur les traits morphologiques et physiologiques. La hauteur finale des plantes, le nombre de feuilles et la distance entre les nœuds ont diminué avec l'altitude, reflétant les contraintes imposées par des températures plus fraîches sur la croissance en longueur. En revanche, le diamètre des tiges, la surface foliaire et la biomasse aérienne (AGB) étaient plus élevés au site de haute altitude (1 700 m), indiquant une tendance vers des tiges plus épaisses et des feuilles plus grandes en haute altitude. Le taux de croissance relative de la surface foliaire (RGRA) a augmenté avec l'altitude, démontrant une efficacité accrue de l'acquisition de carbone dans des environnements plus frais. La densité stomatique a diminué aux altitudes plus élevées, tandis que la longueur des pores a augmenté, suggérant des ajustements compensatoires dans la régulation des échanges gazeux. Une variation phénotypique importante entre les variétés génétiques a confirmé la présence d'une diversité intra-spécifique significative pouvant soutenir les futurs efforts de sélection.

Conclusion – Cette thèse démontre que *C. canephora* présente de fortes réponses phénotypiques à la variation environnementale et que des variations phénotypiques ont eu lieu durant le siècle passé. Les analyses historiques d'herbiers révèlent des

changements significatifs de traits cohérents avec une réponse aux conditions climatiques changeantes tout au long du XX^{e} siècle. Les analyses spatiales contemporaines mettent en évidence une différenciation fonctionnelle étendue à travers l'aire de répartition de l'espèce, reflétant à la fois l'hétérogénéité environnementale et la variabilité des traits au niveau des populations. Les résultats des essais multilocaux confirment une plasticité à court terme prononcée dans la croissance, la morphologie foliaire et la régulation stomatique le long d'un gradient altitudinal, avec des réponses spécifiques à l'origine génétique des plantules. Ces résultats ont des implications directes pour la conservation des ressources génétiques du café sauvage et pour le développement de systèmes de production de café résilients au climat en Afrique centrale. Préserver la diversité génétique et écologique de l'espèce sera essentiel pour assurer sa pérennité face au changement climatique en cours et pour soutenir les futurs efforts de sélection visant à garantir une production de café durable.

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List of abbreviations

AD	Apparent density
AGB	Aboveground biomass
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
ARES	Académie de Recherche et Enseignement Supérieur
BR	Meise Botanic Garden Herbarium
C_{atm}	Atmospheric CO_2 concentration
CEBioS	Capacities for Biodiversity and Sustainable Development
CEC	Cation-exchange capacity
CEDA	Centre for Environmental Data Analysis
CIFOR	Center for International Forestry Research
ClimCoff	Climate adaptation, carbon fixation and sustainable development through Robusta coffee agroforestry in and around Yangambi
CMIP6	Coupled Model Intercomparison Project Phase 6
CRU	Climatic Research Unit
CV	Coefficient of variation
DRC	Democratic Republic of the Congo
ERAIFT	Ecole Régionale Postuniversitaire d'Aménagement et de Gestion intégrés des Forêts et Territoires tropicaux
FAO	Food and Agriculture Organization of the United Nations
FORETS	Formation, Recherche et Environnement dans la Tshopo
FPH	Final plant height
FSD	Final stem diameter
FWO	World Flora Online
g_{cmax}	Maximum diffusive stomatal conductance to CO_2
GHG	Global greenhouse gas
GLMM	Generalized Linear Mixed Model
H_{max}	Maximum height
H_{min}	Minimum plant height
ICRAF	World Agroforestry Centre
ICO	International Coffee Organization
IISD	International Institute for Sustainable Development
INERA	Institut National pour l'Etude et la Recherche Agronomiques
INL	Internode length
IPCC	Intergovernmental Panel on Climate Change

ISFL	Initiative for Sustainable Forest Landscapes
ISOFYS	Isotope Bioscience Laboratory
iWUE	Intrinsic water use efficiency
KP	Kilometer point
KU Leuven	Katholieke Universiteit Leuven
LA	Leaf area
LDM	Leaf dry mass
LMM	Linear mixed-effects model
LN	Leaf number
MAP	Mean annual precipitation
MAT	Mean annual temperature
Meise BG	Meise Botanic Garden
OM	Organic matter
PACODEL	Centre pour le Partenariat et la Coopération au Développement
PASA-NK	Projet d'Appui au Secteur Agricole dans la Province du Nord-Kivu
PCA	Principal Component Analysis
PC	Principal component
pH	Potential of hydrogen
PS	Seasonal precipitation
PTFE	Polytetrafluoroethylene
RBINS	Royal Belgian Institute of Natural Sciences
RGRA	Relative growth rate in leaf area
SEB	Saturation of exchangeable bases
SLA	Specific leaf area
SMA	Standardized major axis regression
SOC	Soil Organic Carbon
SPS	Stomatal pore size
TERRA	Teaching and Research Centre
TS	Seasonal temperature
ULIEGE	Université de Liège
UNIKIS	Université de Kisangani
USD	United States Dollar
USDA	United States Department of Agriculture
WC	Water content
YBI	Yangambi

Chapter 1. General Introduction

1.1. Context and motivation for this thesis

Coffee is one of the most economically significant agricultural commodities worldwide. With an estimated three billion cups consumed each day, coffee is among the most widely consumed beverages globally, surpassed only by water and tea (ICO, 2024). Because of this high and sustained demand, the global coffee industry has evolved into a complex socioeconomic system connecting producers, processors, traders, and consumers across continents. Notably, the sector plays a pivotal role in tropical regions, where millions of smallholder farmers rely on coffee production as a primary source of income and livelihood (Davis et al., 2006 ; IISD, 2022). Consequently, any disruption to coffee production reverberates widely, affecting both local communities and global markets.

Within this global landscape, the Democratic Republic of the Congo (DRC) occupies a distinctive and historically important position. The country is recognized as the primary center of domestication for *Coffea canephora* (robusta coffee), one of the two principal species cultivated at an industrial scale. Owing to its climatic and edaphic heterogeneity, ranging from lowland equatorial forests to high-elevation montane ecosystems in the Eastern part, the DRC provides environmental conditions conducive to the cultivation of both *C. canephora* and *C. arabica*. These two species represent approximately 40 percent and 60 percent of global coffee production, respectively (Davis et al., 2006, 2025). This ecological diversity not only supports wide-ranging coffee production systems but also underpins the country's exceptional reservoir of wild genetic resources, which remain of major evolutionary and agronomic interest (Depecker et al., 2023; Verleysen et al., 2024).

Historically, the Congolese coffee sector experienced substantial expansion and productivity from the mid-20th century up until the late 1970s. During this period, coffee ranked among the nation's leading export commodities, contributing significantly to foreign exchange earnings and rural development. However, beginning in the 1980s, the sector experienced a severe and prolonged collapse. This decline stemmed from a confluence of factors, including political instability, persistent armed conflicts, institutional deterioration, the dismantling of state-led agricultural support structures, and chronic underinvestment in production and processing infrastructure (USDA, 2025). These multidimensional disruptions drastically reduced national production capacity, weakened supply chains, and eroded the competitiveness of Congolese coffee on international markets. The sector's decline also reduced the economic resilience of rural populations formerly dependent

on coffee income, thereby exacerbating poverty and limiting opportunities for sustainable agricultural development.

In addition to these structural and political constraints, contemporary coffee production in the DRC, like other tropical agricultural systems, is increasingly threatened by the intensifying impacts of global climate change. Several studies have demonstrated that coffee cultivation is highly sensitive to climatic variability, particularly shifts in temperature, precipitation patterns, and extreme weather events. Recurring droughts, shortened rainy seasons, rising mean and maximum temperatures, and the accelerated spread of pests and diseases such as the coffee berry borer and fungal pathogens collectively undermine the stability and productivity of coffee agroecosystems (Jaramillo et al., 2011; Bunn et al., 2015; Läderach et al., 2017). These climate-related stressors affect not only yield quantity but also bean quality, fruit development cycles, and physiological functioning, with cascading impacts on incomes of farmers and the long-term viability of production systems. The broader scientific community has repeatedly highlighted the vulnerability of global coffee production to climate change, and multiple predictive modeling studies suggest substantial reductions in climatically suitable areas for cultivation over the coming decades. These projected impacts are particularly severe for *C. arabica*, which requires more restrictive climatic conditions, but significant declines are also expected for *C. canephora*, especially in low-elevation tropical landscapes (Läderach et al., 2017).

To overcome these climate challenges, the DRC retains an underexploited yet exceptional asset: extensive populations of wild *C. canephora* distributed throughout the Congo Basin forests, representing a huge amount of genetic diversity. These wild genotypes, which evolved under diverse ecological conditions, exhibit substantial phenotypic and genotypic variation. Such diversity is likely to harbor adaptive traits of significant agronomic value, including enhanced tolerance to heat stress, improved resilience to water deficits, and greater resistance to emerging pests and pathogens (Davis et al., 2019). Nevertheless, these populations remain poorly characterized, and their potential contribution to breeding programs is largely unrealized. The scientific underrepresentation of these wild resources constitutes a critical bottleneck in efforts to develop climate-resilient coffee cultivars capable of sustaining future production under increasingly unpredictable environmental conditions.

Recent physiological and ecophysiological studies on cultivated *C. canephora* have begun to shed light on the responses of the species to key environmental stressors. Research on elevated temperatures (Martins et al., 2019; Kath et al., 2020, 2021),

water deficit (Semedo et al., 2018; Venancio et al., 2020; Kiwuka et al., 2023; Rodrigues et al., 2024), and increasing atmospheric CO_2 concentrations (Rodrigues et al., 2016; Semedo et al., 2018; Martins et al., 2016) has provided valuable insights into physiological plasticity, photosynthetic adjustments, and the limits of stress tolerance. However, a substantial methodological limitation persists as most of these studies focus solely on cultivated genotypes or improved breeding lines. There is a significant scientific gap concerning the potential responses of wild *C. canephora* to climate change in their native habitats within the Congo Basin. Without systematic ecological, physiological, and genetic assessments of these populations, researchers and breeders lack the empirical basis needed to predict how these lineages may respond to ongoing climatic changes. Moreover, the absence of such information restricts integration of these populations into strategic breeding programs aimed at strengthening the climate resilience of existing cultivars. Given that wild populations often reservoirs of adaptive traits lost during domestication or selection, their neglect represents a substantial missed opportunity in the context of global climate adaptation.

Considering these gaps and the intensifying climate pressures facing coffee production worldwide, there is a need for research focused on the functional, morphological, and ecophysiological variation of wild *C. canephora* traits across the Congo Basin. Such work will provide an empirical foundation for future breeding initiatives targeting improved tolerance to heat, drought, pests, and other stressors expected to intensify under future climate scenarios.

Within this scientific and applied context, the present doctoral thesis aimed to address key knowledge gaps by investigating the ecological and functional variability of wild *C. canephora* traits across multiple environments within its native range. The findings provide critical evidence to support enhanced conservation efforts, as the loss of this diversity would entail a substantial depletion of genetic resources essential for future crop improvement. Moreover, this study offers an important reference for assessing the impacts of climate change on rainforest understory shrubs, a plant functional group that remains largely understudied yet highly vulnerable to both climate change and land-use change. By integrating field-based trait measurements, environmental characterization, and ecophysiological profiling, this research generates robust empirical data that can inform conservation planning, climate adaptation strategies, and the long-term sustainability of coffee production systems in the RDC and beyond.

1.2. Framework of the thesis

This thesis was conducted within the framework of a research program on coffee species in the DRC. The program is led by the Meise Botanic Garden, in partnership with the University of Kisangani, the University of Liège, the INERA, and the CIFOR.

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Through these partnerships, this thesis used herbarium collections from INERA/ Yangambi and Meise BG (Figure 1-1). In parallel, several field missions were carried out in the forests of the Yangambi Biosphere Reserve managed by INERA, as well as in other protected areas in the DRC. Experimental trials were established at two INERA research centers using seeds originating from the living collection of the INERA/Yangambi Coffee Program. The leaf manipulations for functional trait analysis were carried out at the Meise BG. The isotopes were analysed at the ISOFYS laboratory at Ghent University.

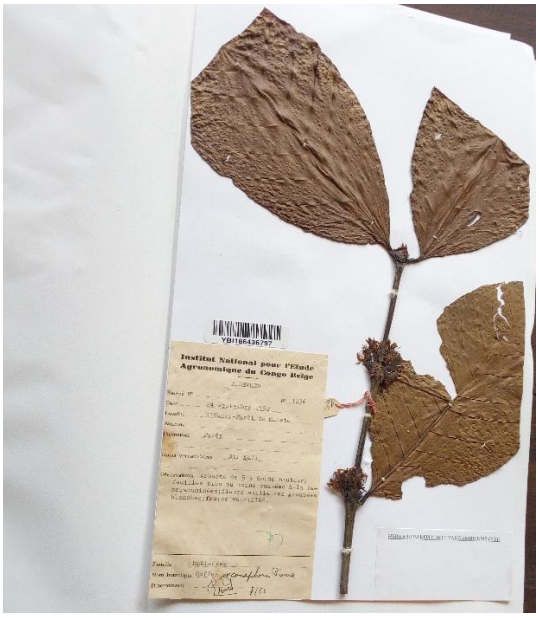

Figure 1-1. Herbarium specimen of *Coffea canephora*, preserved at the Yangambi Herbarium (YBI), Democratic Republic of the Congo.

Chapter 2. Literature review

2.1. Background and rationale

Chapter 2 reviews current knowledge on *C. canephora* in the Congo Basin, integrating its biological, ecological, and agronomic context with broader environmental and climate considerations. It presents the taxonomy, natural distribution, ecology, and genetic diversity of wild populations, highlighting their importance for coffee breeding and conservation. The chapter also examines the historical and current coffee sector in the DRC, including the role of wild and semi-wild populations, and the socio-economic and environmental challenges affecting production. Climate change trends, their impact on tropical forests, and the vulnerability of understory species are discussed, emphasizing the relevance of functional traits, phenotypic plasticity, and resilience. Herbarium-based analyses and stable isotope approaches are presented as tools to study long-term physiological responses and intrinsic water-use efficiency, while environmental gradients and genotype-by-environment interactions illustrate mechanisms underlying trait variation and adaptive capacity. By synthesizing these topics, the chapter identifies key knowledge gaps, particularly the limited understanding of long-term trait dynamics, environmental drivers, and plastic responses in wild *C. canephora*, which justify the integrative research framework adopted in this thesis.

This chapter is mainly based on a manuscript currently under review for publication in *Biotechnologie, Agronomie, Société et Environnement (*BASE*)*:

Hatangi Y., Stoffelen P., Dhed'a B., Vandelook F. and Lassois L. (n.d). *Coffea canephora: A Strategic Resource for Building Resilience and Reviving Coffee Farming in the Democratic Republic of Congo. A review,* submitted on 27 August 2025.

2.2. *Coffea canephora*: biological, ecological and agronomic background

Coffee plants belong to the family Rubiaceae and the genus *Coffea* (Charrier and Berthaud, 1985), which currently comprises 133 scientifically recognized species (Davis et al., 2025). The genus is native to tropical Africa and Madagascar, where species occupy a wide range of ecological niches, from lowland rainforests to montane ecosystems (Davis et al., 2006, 2012). Despite this considerable taxonomic and ecological diversity, global coffee production relies almost exclusively on two species: *Coffea arabica* L. and *Coffea canephora* Pierre ex A. Froehner.

Coffea arabica originated in the highlands of Ethiopia and South Sudan, as well as on the slopes of Mount Marsabit in Kenya, whereas *C. canephora* is native mainly to the lowland humid forests of Central and West Africa, with a natural range extending from Guinea to Uganda and Angola (Davis et al., 2006, 2019). These contrasting evolutionary and ecological origins underpin major differences between the two species in terms of physiology, genetic diversity, and environmental tolerance. At present, arabica accounts for approximately 60% of global coffee production, while robusta represents about 40% (ICO, 2024).

From an evolutionary and genetic perspective, *C. arabica* is an allotetraploid species (2n = 4x = 44) with predominant self-pollination, originating from a hybridization event between the diploid species *C. canephora* and *C. eugenioides* (Lashermes et al., 1999; Maurin et al., 2007). Both parental species still occur naturally in Central and East Africa, notably in the DRC and Uganda (Kiwuka et al., 2021; Depecker et al., 2023). In contrast, *C. canephora* is a diploid, outcrossing species characterized by high genetic diversity and strong population structuring. Genetic studies distinguish two major groups: Guinean and Congolese, further subdivided into six genetic subgroups (Cubry et al., 2013), while agronomic classifications recognize two main cultivated groups: Kouillou (Conilon, SG1) and Robusta sensu stricto (SG2) (Ferrão et al., 2024).

Ecologically, *C. canephora* is a shade-adapted understory species typical of Guineo-Congolian humid tropical forests, where it persists in areas that have not undergone severe deforestation. Wild populations are still documented in several Central and East African countries, including Uganda and the DRC (Kiwuka et al., 2023; Depecker et al., 2023; Hatangi et al., 2023). These populations constitute a key reservoir of genetic and functional diversity and are increasingly recognized as

essential resources for conservation and climate-resilient coffee breeding (Davis et al., 2012; Aerts et al., 2017).

2.3. Coffee cultivation

Coffee cultivation depends on the ecological requirements specific to each species and on farming practices adapted to local conditions. *C. arabica* and *C. canephora* differ considerably in their ecological preferences. Arabica grows best at altitudes between 800 and 2000 m, with optimal temperatures of 18-24 °C. In contrast, robusta traditionally thrives at lower altitudes (<800 m) and at temperatures between 21 and 27.5 °C (DaMatta and Ramalho, 2006; Partelli et al., 2013). Recent studies, however, have shown that *C. canephora* can produce good yields under lower mean annual temperatures of around 20.5 °C, with minimum and maximum temperatures of 16.2 °C and 24.1 °C, respectively (Kath et al., 2020). Observations in the DRC confirm that robusta can grow successfully up to 1000 m, and even as high as 1700 m, although a recent study demonstrated that both temperature and rainfall distribution strongly influence flowering time, potentially causing delays or premature flowering (Kath et al., 2023). Optimal rainfall ranges between 1200 and 2000 mm per year, with a short dry season of two to three months that favors floral induction (Tournebize et al., 2022). Excess rainfall (>3000 mm) reduces productivity and promotes disease. Coffee trees prefer deep, well-drained soils rich in organic matter and slightly acidic, with a pH between 5.5 and 6.5 (DaMatta et al., 2007; Dai et al., 2026). They commonly establish symbiotic associations with arbuscular mycorrhizal fungi (AMF) that enhance nutrient uptake, particularly phosphorus, and improve plant growth and physiological performance (Prates Júnior et al., 2019; Dai et al., 2026).

Establishing plantations involves plowing to a depth of 30-40 cm, applying soil amendments, and transplanting seedlings at the onset of the rainy season (DaMatta et al., 2007). Planting density varies between species: 1600-2000 plants/ha for arabica (2 m × 2.5 m) and 1100-1300 plants/ha for robusta (2.5 m × 3 m). Young coffee trees are vulnerable to strong winds, so windbreaks are recommended around plots. The role of shade has been debated for decades (DaMatta et al., 2007; Jha et al., 2014; Gomes et al., 2020). Although full-sun cultivation has become the norm, moderate shading is now recognized as beneficial for arabica. For robusta, shade is often less necessary, but it can be valuable during periods of water stress. Shade trees lower soil and air temperatures, enrich the soil with organic matter, improve soil structure, enhance functional biodiversity, and provide additional useful products for farmers (Kiyingi and Gwali, 2012; Gomes et al., 2020; Tschora and Cherubini, 2020). The choice of shade species should be made regarding canopy density, growth rate, and

interaction with coffee plants. Regular pruning of shade trees in particular improves water use efficiency (Buyinza et al., 2023).

Plantation management includes weeding, pruning (formative, maintenance, and rejuvenation), mulching, and, in the case of robusta, careful management of suckers, which is especially important. Diseases and pests represent a serious threat to coffee cultivation. Coffee leaf rust (*Hemileia vastatrix*) is the primary disease affecting arabica coffee (Avelino et al., 2015; Berny Mier Y Teran et al., 2025), resulting in annual economic losses estimated at $ 2 billion due to control costs (Van Der Vossen et al., 2015; Zambolim, 2016). It is controlled through sanitary pruning, the use of resistant varieties, and, if necessary, fungicide applications. Robusta, less susceptible to rust, is nevertheless vulnerable to other threats such as coffee wilt disease (*Fusarium xylarioides*) (Hindorf and Omondi, 2011). Among insect pests, the coffee berry borer (*Hypothenemus hampei*) and the green scale (*Toxoptera aurantii*) cause significant damage, necessitating integrated pest management strategies (Jaramillo et al., 2006; Aristizábal et al., 2016).

Harvesting is performed when cherries reach physiological maturity, indicated by a uniform bright red color for arabica or a yellow to dark red color for robusta. The most recommended method is selective hand-picking, which involves harvesting only ripe cherries to ensure coffee quality. After harvest, fruits must be processed quickly using one of three main methods: wet, dry, or semi-wet (Bollen et al., 2025b). In the wet method, cherries are pulped, subjected to controlled fermentation for 12-36 hours depending on temperature, washed, and dried to a moisture content of 10-12%. In the dry method, cherries are spread out on drying beds and turned regularly for two to three weeks. The semi-wet method combines the two, pulping the cherries without full fermentation while retaining part of the mucilage. Once dried, the beans are hulled and then sorted, either mechanically or manually, before being stored in natural fiber bags in well-ventilated, humidity-controlled facilities. For export, packaging follows international standards, notably the use of 60-kg bags.

Coffee production encompasses a gradient of systems reflecting agroecological conditions and producer capacity. In the Congo Basin, traditional extensive systems dominate, with coffee grown under indigenous shade trees and minimal external inputs, supporting biodiversity but resulting in low yields (Tscharntke et al., 2012; Boreux et al., 2016). Semi-intensive systems are expanding in Uganda and parts of the DRC, combining partial shade with moderate input use and basic practices such as pruning and fertilization (Buyinza et al., 2023). Highly intensive systems, typical of Vietnam and Brazil, rely on full-sun monocultures, high planting densities,

irrigation, and substantial agrochemical inputs, achieving high yields but raising sustainability concerns (Perfecto et al., 2005; Jha et al., 2014). Agroforestry and organic models are increasingly promoted as sustainable alternatives that enhance soil health and reduce chemical dependence in response to climate and market pressures.

2.4. Global coffee production

Coffee is produced in more than 80 countries across tropical regions, providing a substantial source of income for over 125 million people (IISD, 2022) (Figure 2-1). In 2024, Brazil remains the world's leading coffee producer, followed by Vietnam and Colombia (FAOSTAT, 2024). Vietnam is notable for its robusta production, while Colombia is renowned for the quality of its high-altitude arabica. Indonesia and Ethiopia complete the top five countries in production.

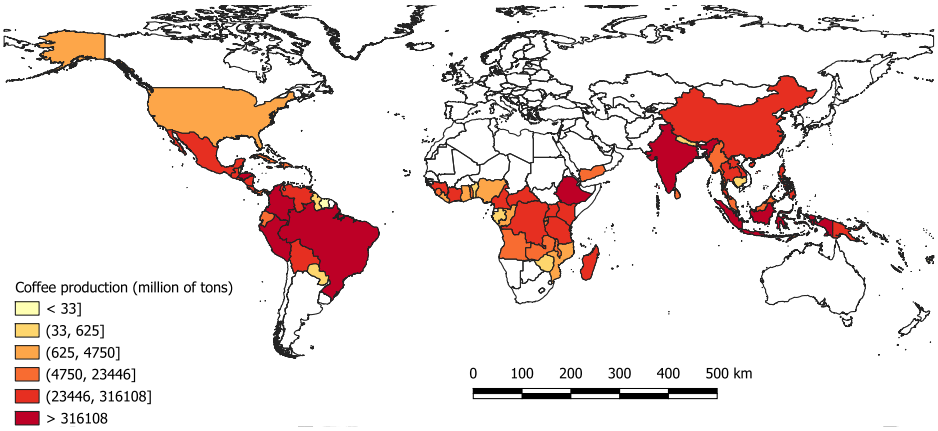

Figure 2-1. Green coffee production by country in 2023. *Source: FAOSTAT (2024)*.

The historical evolution of global coffee production exhibits a general upward trend since the 1960s, with notable fluctuations and several peaks (Figure 2-2). According to FAOSTAT (2024), total production, which stood at about 5 million tons in 1960, nearly doubled to reach 10 million tons in 2022. The first decades, between 1960 and 1980, were characterized by production levels ranging between 3 and 3.5 million tons, followed by a first peak in the late 1970s. A relative decline was observed in the early 1980s, followed by a gradual recovery that led to a second peak around the year 2000, before stabilizing and rising again to reach a historical maximum in 2022. Regional trends highlight contrasting dynamics between continents. In Latin America, production has been established for a long time and has remained relatively stable. Asia, by contrast, has experienced a spectacular expansion since the 1990s: production there increased from less than one million tons in 1980 to about 3.2 million tons in

2022, mainly due to the intensive development of robusta in Vietnam. Africa, however, has followed a very different trajectory: while it produced about one million tons in 1960, representing 30% of global production at that time, today it accounts for only 10-12%. Following a period of stagnation from 1990 to 2010, a recovery has been observed since 2010, with volumes reaching nearly 1.5 million tons in 2022. In Africa, coffee cultivation has played a pivotal role in rural economies for decades, providing essential income and livelihoods for family farms, while supporting local infrastructure and agricultural cooperatives. Ethiopia and Uganda are currently the two leading African producers (ICO, 2024). In Uganda, this dynamic is particularly driven by robusta production, resulting from sustained investments in research, infrastructure, and farmer support programs over the past 30 years (AgriProfits, 2025).

Overall, current data show that global coffee production is marked by strong geographical concentration: Latin America maintains its historical dominance thanks to Brazil and Colombia; Asia has secured a strong position due to Vietnam's rapid growth; and Africa, although it remains a region of origin and a major actor in terms of genetic diversity and wild species represents only a modest share of total production. These trends reflect structural changes in the sector and the growing importance of factors such as technological investment, value chain organization, and access to international markets in shaping the competitiveness of producing countries.

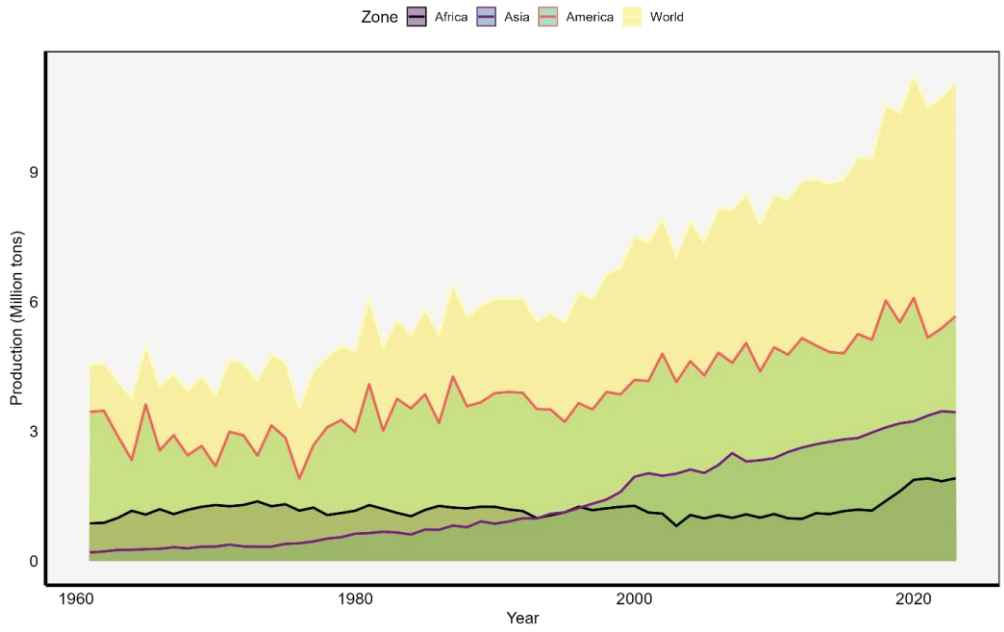

Figure 2-2. Evolution of global green coffee production (in million tons) from 1961 to 2023. Source: FAOSTAT (2024).

2.5. The coffee sector in the Democratic Republic of Congo

The DRC has historically been one of the major coffee-producing countries in Africa, owing to its exceptional diversity of climatic and edaphic conditions that allow the cultivation of both *C. arabica* and *C. canephora*. Throughout the 1970s and 1980s, the country ranked among the leading African exporters of coffee, with annual production approaching 120,000 tons (FAOSTAT, 2024). During this period, robusta dominated national production, while arabica was primarily cultivated in the eastern highlands.

From the early 1990s onward, coffee production in the DRC declined sharply. Political instability, armed conflicts, deterioration of infrastructure, and the collapse of research, extension, and marketing systems led to the abandonment of plantations and the disorganization of value chains (Makanisi, 2025). By the early 2000s, national production had fallen to historically low levels, below 10,000-12,000 tons (FAOSTAT, 2024). Although a modest recovery has been observed since 2010, the DRC has not regained its former position within the African coffee sector.

At present, coffee production in the DRC is estimated at approximately 500,000 bags per year, of which fewer than 200,000 are exported (Figure 2-3), placing the country among secondary African producers (ICO, 2024; USDA, 2025). The sector is dominated by smallholder farmers, who manage many plantations under traditional or semi-intensive production systems, with limited access to inputs, credit, and technical assistance.

Robusta coffee accounts for most of the national production and is cultivated mainly in low- and mid-altitude regions of the northwest, Kasai, and parts of North Kivu, whereas arabica is concentrated in eastern provinces such as North and South Kivu, Ituri, and Haut-Uélé (Makanisi, 2025). Although arabica contributes a smaller share of total volume, it plays a disproportionate role in export value due to the growing demand for specialty coffees from high-altitude terroirs (ICO, 2024). Overall productivity remains low compared with other producing countries (Figure 2-3), reflecting aging plantations, limited genetic improvement, and suboptimal agronomic management.

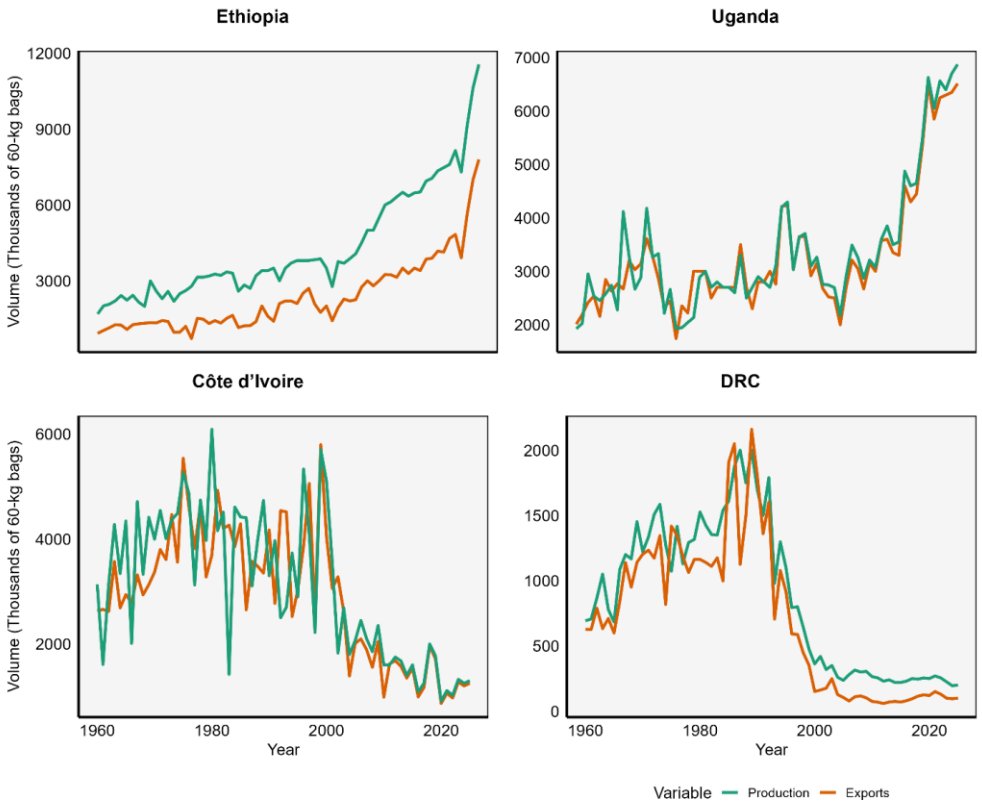

Figure 2-3. Evolution of coffee production and exports, expressed in thousands of 60-kg bags, in four African countries (Ethiopia, Uganda, Côte d'Ivoire, and the DRC) between 1960 and 2024. Source: USDA (2025).

The DRC lies at the core of the natural distribution range of *C. canephora* and harbors extensive wild and semi-wild populations within Guineo-Congolian humid forests (Davis et al., 2006; Depecker et al., 2023). Historically, Congolese genetic resources played a central role in the domestication and global dissemination of robusta coffee, notably through early selection programs conducted in Java during the early twentieth century (Herrera and Lambot, 2017). Today, wild and semi-wild *C. canephora* populations continue to contribute to local production systems, either directly or through introgression into cultivated material. Beyond their immediate agronomic value, these populations constitute a strategic reservoir of genetic and functional diversity, particularly relevant for breeding programs targeting tolerance to heat, drought, and emerging pests and diseases under climate change (Davis et al., 2012; de Aquino et al., 2022). However, their potential remains largely underexploited due to

limited characterization, insufficient conservation measures, and weak integration into national research and development strategies.

The Congolese coffee sector faces multiple, interacting challenges. Climate change represents a major environmental threat, with rising temperatures and increasingly irregular rainfall patterns affecting flowering phenology, fruit development, and yields (Bunn et al., 2015; Kath et al., 2020, 2021, 2022, 2023). Although *C. canephora* is more tolerant to heat and humidity than *C. arabica*, it remains vulnerable to drought stress and rainfall variability, particularly during reproductive stages (Kiwuka et al., 2023).

Deforestation and habitat degradation further threaten wild *C. canephora* populations, increasing the risk of genetic erosion and loss of adaptive potential (Davis et al., 2012; Depecker et al., 2023). At the same time, strong economic constraints, price volatility, low farm-gate prices, weak cooperatives, limited access to markets and infrastructure, undermine producer livelihoods and discourage investment (ICO, 2024). In the DRC, these challenges are exacerbated by prolonged political instability, insufficient institutional support, and the collapse of research and extension services (Makanisi, 2025).

2.6. Climate change in the Congo Basin and implications for tropical forests

The Congo Basin, which hosts the world's second-largest tropical forest, has experienced significant climatic changes over recent decades (Libalah et al., 2026). Observational data show a clear warming trend across Central Africa, with mean annual temperatures increasing by approximately 0.8–1 °C since the mid-twentieth century, a rate comparable to or exceeding the global average (James et al., 2013; IPCC, 2023). This warming affects both lowland and montane regions and is projected to intensify. Ground-based records from sites such as the Yangambi Biosphere Reserve, together with basin-scale analyses, further indicate shifts in rainfall regimes, including changes in precipitation intensity and seasonality (Hatangi et al., 2023; Yakusu et al., 2023). In central parts of the basin, persistent drying trends have been linked to altered atmospheric circulation, notably a weakened Walker circulation and warming of the tropical Atlantic, which reduce moisture convergence and rainfall (Wongchuig et al., 2025). Although total annual rainfall has not declined uniformly across the basin, rainfall variability has increased, with longer dry spells, altered timing of rainy seasons, and more frequent extreme rainfall events (James et al., 2013; Nicholson and Dezfuli, 2013).

These climatic changes are expected to substantially affect the structure, composition, and functioning of Congo Basin forests, which depend on relatively stable thermal and hydrological conditions (Lewis et al., 2009b; Bretfeld et al., 2018; Tng et al., 2018). Rising temperatures increase atmospheric evaporative demand, intensifying plant water stress even in high-rainfall regions, with potential consequences for forest productivity and drought-induced tree mortality (Malhi et al., 2013; Hubau et al., 2020). Concurrent shifts in rainfall regimes may alter soil moisture dynamics, constrain seedling establishment, and modify competitive interactions, with long-term implications for forest regeneration and species composition. These impacts are exacerbated by deforestation and forest degradation, which reduce canopy cover, lower humidity, and amplify edge effects, further weakening ecosystem resilience to climatic extremes (Aleman et al., 2018). Climate change also affects the role of Congo Basin forests in the global carbon cycle, as soil greenhouse gas fluxes are highly sensitive to temperature and moisture controls on CO_2, CH_4, and N_2O emissions, creating potential feedback with regional and global climate systems (Daelman et al., 2025).

Understory plant communities, including shade-adapted species such as *C. canephora*, are particularly vulnerable because they rely on stable microclimatic conditions. Although forest canopies generally buffer understories from thermal and hydric extremes, even modest macroclimatic changes can weaken this buffering and alter local temperature and moisture regimes (De Frenne et al., 2019; Zellweger et al., 2019). Higher temperatures and intensified dry periods can reduce soil water availability and impair photosynthesis, phenology, and reproductive success, especially for species with narrow thermal and hydric niches (Feeley et al., 2011). Limited dispersal capacity and slow demographic rates further restrict the ability of many understory species to track shifting climatic conditions. In addition, climate-driven changes in canopy structure such as increased tree mortality, altered phenology, and more frequent gap formation, can increase light availability, temperature variability, and vapor pressure deficit in the understory, indirectly amplifying climatic stress (Scheffers et al., 2014; Jucker et al., 2018). Despite their importance for forest biodiversity and ecosystem functioning, understory species remain poorly studied in the context of climate change in Central Africa, a critical gap given the ecological and economic significance of wild and semi-wild *Coffea* species as both native forest components and genetic resources for climate-resilient agroforestry and breeding programs.

2.7. Functional traits as indicators of plant responses to climate

Plant functional traits are the measurable morphological, physiological, and phenological characteristics, which mediate how individuals and species capture resources, tolerate stress, and contribute to ecosystem functioning under changing climates (Lavorel and Garnier, 2002; Violle et al., 2007). Trait-based ecology provides a mechanistic framework linking climate drivers (e.g., temperature, precipitation, vapor pressure deficit) to plant performance and vegetation change (Wright et al., 2004; Li and Prentice, 2024).

The leaf economics spectrum (LES) describes a global axis of leaf trait variation ranging from resource-acquisitive to resource-conservative strategies (Wright et al., 2004; Reich, 2014). High specific leaf area (SLA), high nutrient concentrations, and high photosynthetic rates characterize acquisitive leaves, whereas low SLA and dense tissues typify conservative leaves (Wright et al., 2004). These trait syndromes covary with climate and resource availability across biomes, forming a key framework for interpreting changes in leaf traits with environmental gradients, including light and moisture (Li and Prentice, 2024).

The trade-off between carbon gain and water loss is a central organizing principle of plant eco-physiology, as plants regulate stomata to optimize carbon assimilation while minimizing water loss. At the leaf level, CO_2 assimilation (A) and transpiration (E) are coupled through stomatal conductance (g_s), such that increases in carbon gain generally entail greater water loss. Intrinsic water-use efficiency (iWUE), defined as A/g_s, captures this balance independently of atmospheric vapor pressure deficit. According to stomatal optimization theory, g_s is regulated to maximize carbon assimilation per unit water cost over time (Cowan, 1978), but this optimization is constrained by plant hydraulics: increasing transpiration lowers leaf water potential and elevates the risk of xylem embolism (Sperry et al., 2002, 2007). Species differ in how closely they operate to hydraulic xylem failure thresholds, reflecting contrasting stomatal regulation strategies and hydraulic safety margins, here defined as the difference between minimum seasonal water potential (Ψ_min) and the xylem water potential causing 50% loss of conductivity (P50) (Meinzer et al., 2009). Species operating closer to these limits tend to maintain higher g_s and potentially higher A, but at the cost of increased hydraulic risk, consistent with a safety-efficiency trade-off (Henry et al., 2019). In contrast, more conservative species restrict g_s to preserve hydraulic integrity, which can enhance intrinsic water-use efficiency (iWUE) through

the coupling between A and g_s, as predicted by stomatal optimization theory (Medlyn et al., 2011) and supported by isotopic evidence (Seibt et al., 2008).

Variation in stomatal density, stomatal size, and especially maximum stomatal aperture directly determines maximum diffusive conductance (g_{smax}) and the speed and amplitude of stomatal responses, thereby shaping both peak assimilation rates and transpirational capacity (Franks and Beerling, 2009).

These physiological controls are often reflected in coordinated leaf trait syndromes. Specific leaf area (SLA) is tightly linked to carbon–water trade-offs: high SLA leaves are typically thinner, less dense, and associated with higher photosynthetic capacity and g_s, favoring rapid carbon gain but often lower iWUE and shorter lifespan (Wright et al., 2004; Reich, 2014). In contrast, low SLA leaves are structurally reinforced, exhibit lower g_s and transpiration rates, and tend to support higher iWUE and greater drought resistance (Wright et al., 2004; Poorter et al., 2009). This pattern aligns with the leaf economics spectrum (LES), where acquisitive species (high SLA, high nitrogen, large stomatal apertures, high A) occupy the 'fast-return' end, and conservative species (low SLA, reduced stomatal aperture, lower g_s, longer leaf lifespan) occupy the 'slow-return' end (Wright et al., 2004; Sack and Scoffoni, 2013). Increasing evidence indicates that stomatal traits (density, size, aperture regulation), SLA, and hydraulic traits such as embolism resistance and turgor loss point are coordinated along environmental gradients, reinforcing the concept that iWUE emerges from multiscale integration of leaf structure, stomatal anatomy, and xylem safety-efficiency trade-offs (Bartlett et al., 2016; Anderegg et al., 2018).

Tropical forest understory species are adapted to chronically low irradiance, buffered thermal regimes, and spatially heterogeneous soil moisture, conditions that favor high specific leaf area (SLA), low leaf mass per area (LMA), and extended leaf lifespan to maximize carbon return under light limitation. Recent trait-based studies across Amazonian and Southeast Asian forests show substantial intraspecific plasticity in SLA, chlorophyll concentration, stomatal density, and photosynthetic capacity along fine-scale light gradients generated by canopy gaps and vertical stratification (Umaña et al., 2018; Fyllas et al., 2020). Experimental drought manipulations further demonstrate adjustments in water-use traits, including stomatal sensitivity and turgor loss point, indicating coordinated shifts in carbon–water balance under increasing moisture stress (Rowland et al., 2015; Anderegg et al., 2018). Long-term trait comparisons between historic herbarium specimens and recent collections in the Congo Basin reveal increased leaf size and SLA in understory woody shrubs, likely

related to warming and elevated CO_2, illustrating how trait analyses detect climate responses (Hatangi et al., 2023).

Trait-based approaches provide a powerful framework for assessing plant resilience, the capacity to resist, tolerate, or recover from climatic stress, by linking functional traits to ecological outcomes and demographic performance (Violle et al., 2007; Díaz et al., 2016). At the community level, metrics such as community-weighted means (CWMs) and functional diversity capture dominant strategies and the range of trait variation, revealing how assemblages buffer environmental change; for example, CWMs of leaf traits shift along aridity gradients and drought experiments, and high functional diversity can reduce biomass loss under extreme conditions, particularly in tropical forests (Langan et al., 2025; Serrano-León et al., 2025). At the species level, analyses of leaf economic traits (specific leaf area, leaf nitrogen, leaf lifespan), hydraulic traits (turgor loss point, embolism resistance, stomatal density and aperture), and root traits (rooting depth, root tissue density) identify the physiological and morphological characteristics that confer resilience or vulnerability under stress (Fyllas et al., 2020; Li and Prentice, 2024). Linking these traits to performance metrics such as growth, survival, or reproduction reveals how species with conservative water-use and high hydraulic safety maintain function during drought, while acquisitive species with high SLA and stomatal conductance perform better under favorable conditions. Integrating species- and community-level trait data thus provides a mechanistic understanding of ecosystem resilience and enables predictive modeling of vegetation responses to climate change.

2.8. Resilience, adaptation and phenotypic plasticity in plants

Resilience in ecological systems refers to the capacity to absorb disturbance while maintaining essential structure and function (Holling, 1973; Wohl et al., 2024). In plant biology, resilience encompasses resistance to stress, recovery after disturbance, and long-term persistence under environmental change (Lloret et al., 2011; Reich, 2014). Phenotypic plasticity, the ability of a genotype to express different phenotypes across environments, enables plants to respond rapidly to climatic variability (Bradshaw, 1965; Nicotra et al., 2010a; Sommer, 2020; Chen et al., 2024). Plasticity allows plants to adjust morphology, physiology, and phenology in situ, providing immediate buffering against fluctuating environments (Nicotra et al., 2010a; Stotz et al., 2021; Laitinen, 2024). In contrast, local adaptation refers to the heritable evolutionary differentiation of populations that increases fitness in their native environment, often leading to trait divergence along climatic or environmental

gradients (Nicotra et al., 2010a; Valladares et al., 2014; Liu et al., 2020). Together, plastic and genetic processes shape plant resilience across temporal scales.

Plasticity enables plants to adjust functional traits such as specific leaf area, stomatal conductance, biomass allocation, and phenology, in response to temperature and moisture variability (Poorter et al., 2009; Nicotra et al., 2010a). Global syntheses show that environmental heterogeneity promotes greater plastic responsiveness, particularly in regions with strong climatic variability (Nicotra et al., 2010a; Chen et al., 2024). These trait adjustments can stabilize carbon gain and water-use efficiency under moderate drought or heat stress, enhancing short-term resilience (Reich, 2014; Laitinen, 2024). However, plastic responses are context-dependent, may incur physiological costs, and can fail under extreme conditions (Ghalambor et al., 2007; Nicotra et al., 2010a). Maladaptive plasticity may also arise when environmental cues no longer reliably predict selective pressures, a situation increasingly likely under rapid climate change (Ghalambor et al., 2007; IPCC, 2023). Thus, while plasticity can buffer populations in the short term, it does not replace the need for evolutionary adaptation to ensure long-term persistence.

Local adaptation occurs when populations exhibit genetically based trait differences that confer higher fitness in their native environments compared to foreign environments (Kawecki and Ebert, 2004). Such differentiation is common along altitudinal and moisture gradients, where divergent selection drives shift in phenology, water-use efficiency, and growth-related traits (Aitken et al., 2008; Leinonen et al., 2013).

Recent empirical studies confirm that climatic gradients shape both phenotypic and genomic differentiation (De-la-Cruz et al., 2025). Genomic analyses of desert plant populations similarly identify climate-associated allelic variation linked to adaptive trait divergence (Zhang et al., 2024). These findings demonstrate that baseline trait distributions often reflect historical selection pressures, while plasticity modulates trait expression under current conditions (Leinonen et al., 2013; Stotz et al., 2021). Climate change may disrupt historical genotype-by-environment matching, increasing the risk of maladaptation if environmental conditions shift beyond the range of past selection (Aitken et al., 2008; IPCC, 2023). Consequently, standing genetic variation within populations remains a critical determinant of long-term resilience.

2.9. Using herbarium specimens to study long-term plant responses to climate change

Herbarium specimens serve as invaluable historical records, preserving morphological, phenological, and distributional information across centuries (Lavoie, 2013; Meineke et al., 2018). By providing time-stamped snapshots of plant traits and occurrences, herbarium collections allow researchers to reconstruct past environmental conditions and assess long-term ecological and evolutionary responses to climate variability (Willis et al., 2017; Daru et al., 2018). Advances in digitization and imaging, combined with trait extraction techniques, have greatly expanded the utility of these collections for quantitative analyses of plant responses to global change (Heberling and Isaac, 2017; Meineke et al., 2018).

Herbarium-based studies have provided strong evidence that plant traits and phenology respond to climate change (Ouédraogo et al., 2020). Analyses of flowering times across decades reveal consistent advances in phenology in response to rising temperatures (Davis et al., 2015). Similarly, changes in leaf morphology, such as reductions in leaf size or adjustments in stomatal density, have been documented and linked to increased temperature and altered precipitation regimes (Willis et al., 2017). These long-term datasets complement experimental and observational studies, providing unique temporal depth that is otherwise impossible to achieve, and allow detection of gradual evolutionary or plastic shifts in plant populations over centuries (Daru et al., 2018; Meineke et al., 2018).

Despite their value, herbarium studies are subject to methodological limitations and potential biases. Specimen collection is often non-random, with overrepresentation of certain species, geographic areas, or flowering stages, which can affect trait-based analyses (Tegelberg et al., 2014; Daru et al., 2018). Preservation techniques may also alter some traits, such as leaf thickness or color, and introduce measurement errors if not properly accounted for (Lavoie, 2013; Heberling and Isaac, 2017). Furthermore, temporal and spatial gaps in collections can limit the ability to detect fine-scale climate responses, necessitating careful statistical treatment and validation against independent datasets (Willis et al., 2017; Meineke et al., 2018). Despite these challenges, herbarium specimens remain a uniquely powerful resource for studying historical plant responses to climate change, providing insights into both phenotypic plasticity and evolutionary adaptation over timescales inaccessible to conventional ecological monitoring.

2.10. Stable isotopes and intrinsic water-use efficiency in plant ecophysiology

Stable isotopes are non-radioactive forms of an element differing in atomic mass. In plant ecophysiology, the ratio of stable carbon isotopes ($^{13}C/^{12}C$), expressed as $\delta^{13}C$ (‰) relative to an international standard, provides an integrative indicator of photosynthetic carbon assimilation. In C_3 plants, carbon isotope discrimination ($\Delta^{13}C$) occurs because Rubisco preferentially fixes ^{12}C over ^{13}C during photosynthesis (Farquhar et al., 1982, 1989).

Carbon isotope discrimination ($\Delta^{13}C$) is primarily determined by the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a), which reflects the balance between stomatal conductance (g_s) and net photosynthetic assimilation (A). When stomata are more open, C_i increases and approaches the atmospheric concentration (Ca), leading to greater discrimination against ^{13}C and thus more negative $\delta^{13}C$ values. Conversely, partial stomatal closure reduces C_i, limits $\Delta^{13}C$, and results in less negative $\delta^{13}C$. Because C_i/C_a is functionally linked to the ratio A/g_s (iWUE), $\delta^{13}C$ provides a time-integrated proxy of intrinsic water-use efficiency (iWUE), defined as carbon gained per unit water lost. Therefore, less negative $\delta^{13}C$ values indicate higher iWUE (Seibt et al., 2008; Cernusak et al., 2013). Less negative $\delta^{13}C$ indicates higher iWUE. Long-term studies show rising $\delta^{13}C$-derived iWUE in forests, mainly due to partial stomatal closure under elevated CO_2 (Saurer et al., 2004; Keenan et al., 2013). Interpretation of $\delta^{13}C$ is strengthened when combined with $\delta^{18}O$ in the dual-isotope framework (Scheidegger et al., 2000; Barnard et al., 2012).

The ratio of stable oxygen isotopes ($^{18}O/^{16}O$), expressed as $\delta^{18}O$ (‰), in plant organic matter reflects isotopic enrichment processes occurring during transpiration. Oxygen has two stable isotopes of primary relevance in plant physiology: ^{16}O and ^{18}O. During transpiration, lighter ^{16}O preferentially evaporates, enriching leaf water in ^{18}O, a process called evaporative enrichment. This is governed by VPD, stomatal conductance, atmospheric humidity, and boundary layer resistance (Barbour, 2007; Cernusak et al., 2016). Leaf $\delta^{18}O$ is incorporated into organic molecules, preserving information about transpiration during leaf formation.

Scheidegger et al. (2000) proposed a conceptual dual-isotope model linking $\delta^{13}C$ and $\delta^{18}O$ to stomatal conductance and photosynthetic capacity. In this framework, (1) concurrent increases indicate reduced g_s (stomatal limitation), (2) $\delta^{13}C$ shifts without $\delta^{18}O$ changes indicate altered photosynthetic capacity (biochemical limitation), and (3) divergent patterns reflect coordinated but asymmetric regulation of A and g_s. This

framework has been validated under field conditions (Barnard et al., 2012) and is widely used in ecophysiology studies to interpret plant carbon-water relations.

Intrinsic water-use efficiency is influenced by atmospheric CO_2 (C_a), VPD, and soil moisture. Rising C_a can increase iWUE by allowing partial stomatal closure while maintaining assimilation (Saurer et al., 2004; Keenan et al., 2013). Higher VPD promotes stomatal regulation, increasing both $\delta^{13}C$ and $\delta^{18}O$ (Scheidegger et al., 2000; Barnard et al., 2012; Grossiord et al., 2020). Dual $\delta^{13}C$-$\delta^{18}O$ analysis provides a mechanistic tool to distinguish whether iWUE shifts are driven by stomatal regulation or changes in photosynthetic capacity, enabling robust interpretation of plant responses to environmental stress.

Stable isotopes are widely used to reconstruct long-term physiological responses in forests and crops. In tropical forests, increases in iWUE over recent decades have been inferred from $\delta^{13}C$ in tree rings and leaves, reflecting rising C_a, warming, and increasing VPD (Brienen et al., 2017; Hubau et al., 2020). Understory species experience buffered microclimates with lower irradiance and moderated temperature and humidity, but canopy disturbance or warming can weaken this effect, raising VPD and altering g_s (De Frenne et al., 2019). The dual-isotope model (Scheidegger et al., 2000) and its field application (Barnard et al., 2012) enable separation of stomatal and biochemical controls: parallel $\delta^{13}C$–$\delta^{18}O$ enrichment indicates stomatal limitation, whereas $\delta^{13}C$ shifts alone indicate biochemical limitation.

In perennial crops, $\delta^{13}C$ serves as an integrative drought-tolerance metric, capturing cumulative carbon-water balance during leaf development (Condon, 2004). When combined with $\delta^{18}O$, it can distinguish genotypic variation arising from conservative stomatal regulation versus enhanced photosynthetic capacity, supporting breeding for climate resilience (Scheidegger et al., 2000; Barnard et al., 2012). Overall, dual-isotope analysis provides a robust framework for quantifying plant carbon-water relations and assessing adaptive responses to climate change.

2.11. Environmental and altitudinal gradients as natural experiments

Environmental gradients, systematic changes in abiotic factors such as temperature, humidity, and irradiance across space across space, act as ‘natural experiments’ that reveal how plants adjust their traits under varying conditions (Jump and Peñuelas, 2005; Liu et al., 2020). Altitudinal gradients are particularly informative because they combine predictable shifts in temperature, humidity, and atmospheric pressure over

short spatial scales, while other environmental variables such as soil type often remain relatively constant (Körner, 2007).

Plants growing along these gradients exhibit variation in morphological, physiological, and phenological traits, reflecting both phenotypic plasticity, and local adaptation (Nicotra et al., 2010a; Valladares et al., 2014; Liu et al., 2020). Environmental gradients therefore provide a robust framework for investigating mechanisms underlying trait variation, resource-use strategies, and resilience in understory and perennial species.

Phenotypic variation is determined by both genetic differences and environmental conditions. Genotype-by-environment (G×E) interactions occur when different genotypes respond differently to the same environmental conditions, producing variation in trait expression (Via et al., 1995; Des Marais et al., 2013). In perennial crops, where individual plants persist for multiple years, G×E interactions are particularly important because genotypes experience fluctuating conditions of temperature, light, and soil moisture across seasons. These interactions shape key functional traits and determine which genotypes maintain consistent performance or exhibit beneficial plastic responses under variable environments (Via et al., 1995; Nicotra et al., 2010a; Des Marais et al., 2013). G×E can manifest as plasticity in leaf morphology, stomatal density, photosynthetic capacity, or phenology. Understanding G×E is critical for breeding and conservation, as it identifies genotypes capable of sustaining performance or expressing adaptive plasticity under environmental variability (Sarzynski et al., 2023).

Phenotypic plasticity contributes to resilience by allowing plants to adjust traits rapidly to changes in temperature, vapor pressure deficit (VPD), or light, buffering short-term climate variability (Poorter et al., 2009; Nicotra et al., 2010a). However, plasticity has limits: extreme or novel conditions may exceed the range of trait adjustment, resulting in maladaptation, where trait changes reduce fitness (Ghalambor et al., 2007). Integrating knowledge of plasticity with G×E interactions is therefore crucial for predicting which populations or genotypes are likely to persist under climate change. This information informs strategies for assisted selection, germplasm conservation, and adaptive management (Aitken et al., 2008; Laitinen, 2024). Long-term monitoring along environmental gradients enables quantification of trait shifts and provides mechanistic insight into species' resilience under changing climates (Valladares et al., 2014; Silva et al., 2024).

2.12. Knowledge gaps and justification of the present thesis

Considerable research has documented the ecology, genetics, and agronomy of *C. canephora* within its native range in the Congo Basin, particularly in the DRC. Wild and semi-wild populations in the Guineo-Congolian forests maintain high genetic diversity and contribute to cultivated gene pools (Kiwuka, 2020; Depecker et al., 2023; Verleysen et al., 2023). Morphological and functional traits such as leaf size, stomatal density, and growth rates exhibit both phenotypic plasticity and adaptation to environmental gradients (Silva et al., 2024). Furthermore, sensory and agronomic evaluations have characterized variation among cultivated and wild genotypes, highlighting diversity relevant for breeding and climate resilience (Bollen et al., 2024, 2025a, 2025b).

However, existing research remains fragmented. Genetic structure, sensory quality, and agro-morphological traits have largely been examined independently, with limited integration of functional physiology, environmental gradients, and genotypic background. Long-term trait responses to historical climate change have not been explicitly quantified using retrospective approaches such as herbarium analyses. Moreover, the extent to which genotype-by-environment (G×E) interactions shape trait expression and performance under fluctuating tropical climates remains insufficiently resolved in perennial systems (Nicotra et al., 2010a; Des Marais et al., 2013).

In the Congo Basin, several critical knowledge gaps constrain conservation and climate-adaptive breeding strategies. First, long-term phenotypic responses of woody understory species, including wild *C. canephora*, to past climate change remain poorly documented. Without temporal baselines derived from historical collections, it is difficult to determine whether observed variation reflects short-term plasticity, directional shifts over decades, or stable trait conservatism. Second, although genebanks such as the INERA Yangambi collection have been assessed for agro-morphological and genetic diversity, their functional traits and water-use strategies remain largely unexplored. Third, experimental evidence of phenotypic plasticity in wild *C. canephora* is limited. Seedling stages are critical for establishment and long-term persistence, yet controlled assessments of growth, morphological, and stomatal responses to contrasting environmental conditions, particularly along altitudinal gradients representative of future climate scenarios, are lacking. Finally, the integration of functional traits, physiological performance, and genetic background across environmental gradients remains incomplete. This limits predictive capacity

for identifying genotypes capable of maintaining performance under projected climate change.

This thesis addresses these knowledge gaps through a multi-scale and integrative framework aimed at assessing the functional resilience of wild *C. canephora* to climate change in the Congo Basin. First, it quantifies long-term changes in leaf functional traits of woody understory species using historical herbarium specimens combined with recent field collections, thereby providing empirical evidence of potential phenotypic responses to past climatic shifts. Second, it characterizes the spatial and temporal variability of leaf functional traits and intrinsic water-use efficiency across the DRC and identifies the climatic drivers underlying this variation. By linking trait expression to environmental gradients, the study clarifies carbon-water trade-offs and the ecological determinants of functional performance. Third, it experimentally evaluates phenotypic plasticity in *C. canephora* seedlings by assessing growth, morphological, and stomatal responses to contrasting environmental conditions along an altitudinal gradient. This approach enables direct assessment of environmentally induced trait variation and G×E effects.

By integrating retrospective analyses, spatial assessments, and experimental approaches, this thesis establishes mechanistic links between genotype, phenotype, and environment. It thereby provides a comprehensive basis for understanding climate resilience in wild *C. canephora* and supports conservation and climate-adaptive breeding strategies across the Congo Basin.

Chapter 3. Research questions, objectives, and hypotheses

3.1. Background rationale

The literature review presents current knowledge on *C. canephora* in the Congo Basin, highlighting its ecological, genetic, and agronomic importance. While cultivated coffee has been well studied, wild populations remain poorly understood, particularly regarding functional trait dynamics, spatial differentiation, and phenotypic plasticity. Climate change and habitat degradation threaten these populations and their adaptive potential. This thesis addresses these gaps using historical herbarium analyses, field surveys, and seedling experiments. The study aims to link genotype, phenotype, and environment to inform conservation and climate-resilient breeding strategies. To address these gaps, this thesis adopts an integrative, multi-scale research framework combining historical, spatial, and experimental approaches, which are detailed in this chapter on research questions and objectives.

3.2. Research questions

The overarching research question guiding this thesis is:

How resilient is wild *C. canephora* to climate change across its native range in the Congo Basin, and through which functional mechanisms is this resilience expressed?

This overarching question is addressed through the following specific research questions:

1. Have the leaf functional traits of woody understory species, including *C. canephora*, changed over the past decades in response to recent climate change in the Congo Basin?

   (Addressed in Chapter 4 – Temporal dynamics)

2. Does *C. canephora* exhibit spatial and century-scale temporal variation in leaf functional traits and intrinsic water-use efficiency (iWUE) across its native range in the DRC?

   (Addressed in Chapter 5 – Spatiotemporal variability)

3. How do growth, morphological, and stomatal traits of *C. canephora* respond to contrasting environmental conditions along an altitudinal gradient, and how do these responses vary among genetic origins?

   (Addressed in Chapter 6 – Plasticity along gradients)

3.3. Research objectives

The overall aim of this thesis is to assess the functional resilience of wild *C. canephora* to climate change in the Congo Basin. To achieve this aim, the following specific objectives were defined:

1. To assess long-term changes in leaf functional traits of woody understory species using historical herbarium specimens and recent field collections, thereby assessing potential phenotypic responses to past climate change.

2. To quantify the spatial and century-scale temporal variability of leaf functional traits and intrinsic water-use efficiency (iWUE) in *C. canephora* across the DRC, and to identify the environmental drivers underlying this variation.

3. To experimentally evaluate the phenotypic plasticity of *C. canephora* seedlings by assessing growth, morphological, and stomatal trait responses to contrasting environmental conditions along an altitudinal gradient.

3.4. Research hypotheses

The central hypothesis of this thesis posits that Wild *C. canephora* is functionally resilient to climate change across its native range in the Congo Basin, and this resilience is expressed through coordinated temporal shifts in leaf functional traits, spatial and temporal adjustments in intrinsic water-use efficiency (iWUE), and genetically structured phenotypic plasticity along environmental gradients.

From this central hypothesis, three specific hypotheses, corresponding to the three specific research questions and objectives are formulated:

H1. Leaf functional traits of woody understory species, including *C. canephora*, have varied over recent decades in response to recent climate change in the Congo Basin.

(Temporal response – Tested in Chapter 4)

H2. Wild *C. canephora* exhibits significant spatial and temporal variation in leaf functional traits and intrinsic water-use efficiency (iWUE) across its native range in the DRC, driven by environmental gradients.

(Spatiotemporal differentiation – Tested in Chapter 5)

H3. Growth, morphological, and stomatal traits of *C. canephora* respond plastically to contrasting environmental conditions along an altitudinal gradient, and the magnitude and direction of these responses differ among genetic origins, indicating intraspecific variation in adaptive capacity and potential local adaptation to climatic conditions.

(Plasticity and genetic variation – Tested in Chapter 6)

3.5. Structure of the thesis

This thesis is structured into seven chapters: a general introduction **(Chapter 1)**, the literature review **(Chapter 2)**, Research questions and objectives **(Chapter 3)**, three chapters presenting the main research results **(Chapters 4–6),** and a general discussion **(Chapter 7)**, followed by a general conclusion.

Chapter 1 provides the context of thesis; **Chapter 2** gives a comprehensive synthesis of current knowledge on *C. canephora* in the DRC, thereby situating the research within its broader scientific and regional context. **Chapter 4** investigates long-term

changes in leaf traits of five understory woody species of the Yangambi Biosphere Reserve, including *C. canephora* using a diachronic herbarium-based approach. **Chapter 5** explores spatial and century-scale temporal variation in functional traits and water-use strategies of *C. canephora* across the DRC. **Chapter 6** examines phenotypic plasticity and genotype-by-environment interactions through a multi-location trial conducted along an altitudinal gradient. Together, these chapters provide an integrated assessment of the mechanisms underlying the functional resilience of wild *C. canephora* to climate change, which are synthesized and discussed in the final chapter (**Chapter 7**).

The thesis concludes with a general discussion integrating results across all research chapters and highlighting their implications for understanding the resilience of *C. canephora* under changing environmental conditions (**Chapter 7**). A general conclusion synthesizes the main contributions of the work and outlines recommendations for future research on the adaptive responses of *C. canephora* in the Congo Basin.

3.6. Conceptual outline of the thesis structure

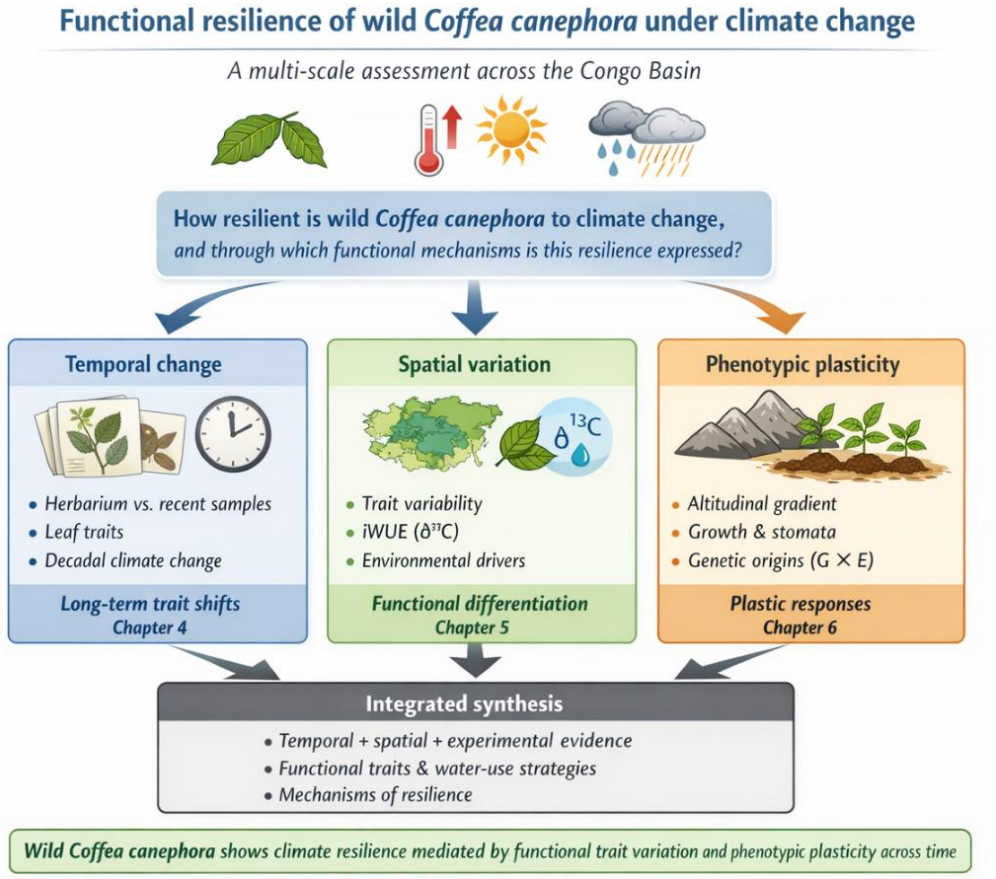

Figure 3-1. Graphical abstract illustrating the multi-scale framework used to assess the functional resilience of wild *Coffea canephora* to climate change in the Congo Basin. Figure generated with assistance from ChatGPT (OpenAI).

Chapter 4. Leaf traits of understory woody species in the Congo Basin forests changed over a 60-year period

4.1. Background and rationale

The previous chapter presented the research questions, the objectives and the structure of this thesis. In this chapter 4, we focus on the leaf physiology of *C. canephora* and other woody species coexisting in the understory of Congo Basin forests. Specifically, we examine how climate change may have influenced leaf functional traits, which serve as key indicators of plant physiological responses to environmental variations.

The study employs a diachronic approach, combining the analysis of leaves from herbarium specimens collected before 1960 with leaves recently collected between 2019 and 2022 in the Yangambi Biosphere Reserve (DRC). Five representative understory species, including *C. canephora*, were selected to assess changes in leaf traits over approximately 60 years. The traits analyzed include specific leaf area, stomatal density, and stomatal and pore length and width.

Two primary objectives guide this chapter. The first is to determine whether leaf functional traits vary according to leaf position within the crown of understory shrubs, in an environment characterized by relatively homogeneous light conditions. The second is to evaluate whether these traits have varied significantly over the past six decades in response to climate change in the Congo Basin. Climatic data from the Yangambi region indicate a significant increase in mean annual temperature since 1961, whereas precipitation shows no clear long-term trend. In this context, analyzing

leaf functional traits provides insight into the phenotypic plasticity of understory species and their capacity to adapt to changing environmental conditions.

By focusing on understory woody species, which remain understudied compared to canopy trees, this chapter fills an important gap in literature. It provides new evidence on the resilience and sensitivity of these species to climate change in African tropical forests and contributes to a better understanding of how tropical forest ecosystems respond to global environmental change.

The content of this chapter is based on the following publication:

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Minor adaptations have been made to comply with university style guidelines.

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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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4.2. 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 analyze 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

4.3. Introduction

Tropical forests are characterized by their high diversity of woody species (Ter Steege et al., 2013; Kearsley et al., 2017; Rahman et al., 2019a) 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., 2015). Understanding species and phenotypic diversity of tropical forest plants is essential to predict effects of climate change on tropical forests and to 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., 2009b; 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 21^{th} 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 (Li 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 (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 center 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 characterized 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 anthropization, 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 analyze 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 analyzed 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.

4.4. Material and methods

4.4.1. 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 Ya Igugu, 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 (classified as Oxisols in the USDA Soil Taxonomy), 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 (Ebuy et al., 2016). They are acidic with a pH between 3 and 4. The vegetation of Yangambi is characterized by a mixture of old-growth and regrowth forests (Tarelkin et al., 2016).

4.4.2. 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 4-1). 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, each separated by at least 1 km, 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 an 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, 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) and for which collection date information was available were selected from the Yangambi (YBI) and the Meise Botanic Garden (BR) herbaria. From each herbarium specimen, a leaf was sampled while taking care not to destroy the voucher. All leaves used in this study were originally collected in the Yangambi region according to their labels.

Table 4-1. 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

4.4.3. 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 colorless 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 mm^2. 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.

4.4.4. 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. (2023) and supplementary material 2-2 for details).

Changes in mean annual temperature and rainfall in Yangambi over the past 60 years were highlighted by analyzing 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, 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. 4-1A). 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. 4-1B).

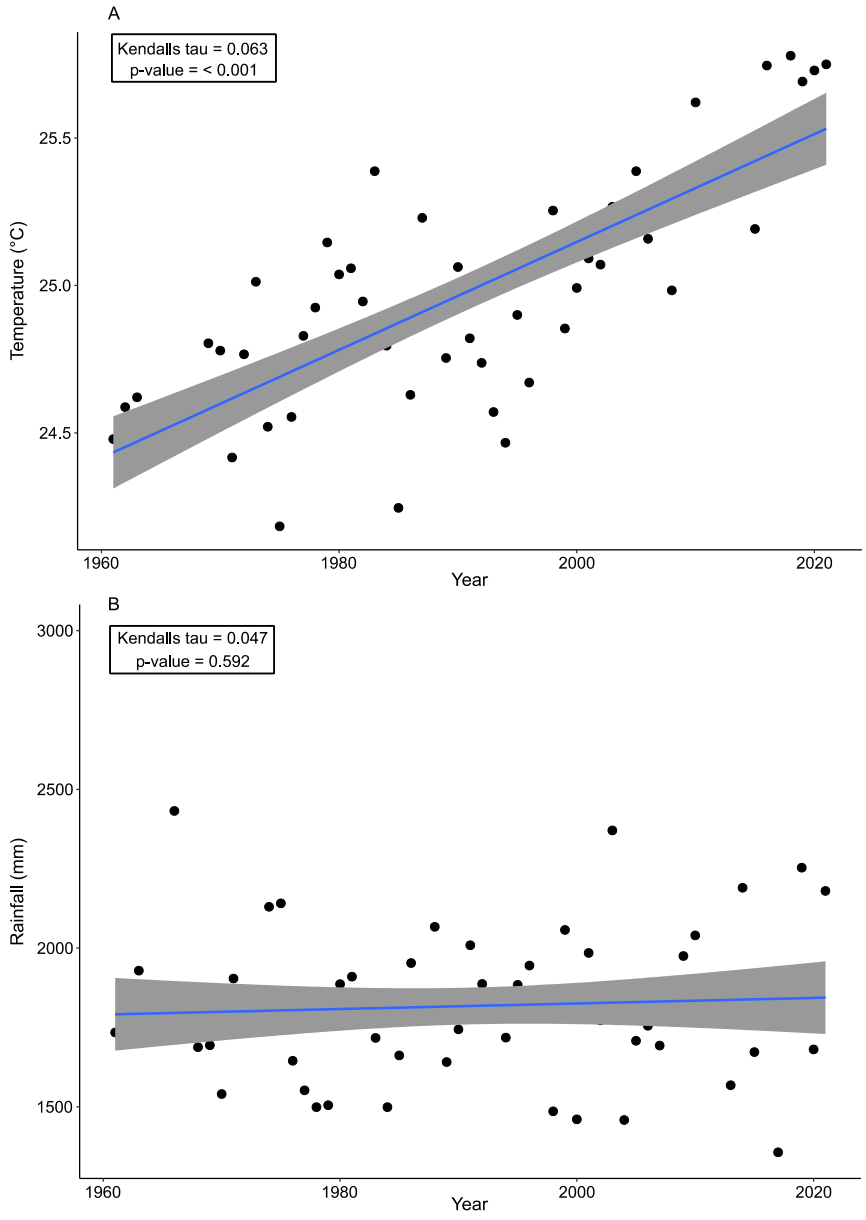

Figure 4-1. Annual mean temperature (**A**) and rainfall (**B**) recorded at Yangambi from 1961 to 2021 (raw data received from INERA, Yangambi, see Yakusu et al. (2023) for details).

4.4.5. Data analysis

All data were analyzed 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 tests 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, 2019). 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.

4.5. Results

4.5.1. 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 4-2). We did, however, find significant differences between species for all leaf traits.

Table 4-2. 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

Coffea canephora large leaves characterized by both high leaf area and high dry mass (Fig. 4-2A, B), while *Hua gabonii* had the smallest leaves (Fig. 4-2A). 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. 4-2C). There was no significant difference in stomatal size measured for leaves collected at different positions in the crown (Table 4-2). Indeed, leaf stomata had lengths around 23.43 ± 6.38 µm regardless of the position of the leaves in the crown (Fig. 4-2D), whereas their widths oscillated around 15.87 ± 4.82 µm on average (Fig. 4-2E). 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. 4-2F). Similarly, the stomatal pore width of all species studied was distributed around the mean value 7.17 ± 2.26 µm (Fig. 4-2G). *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. 4-2I).

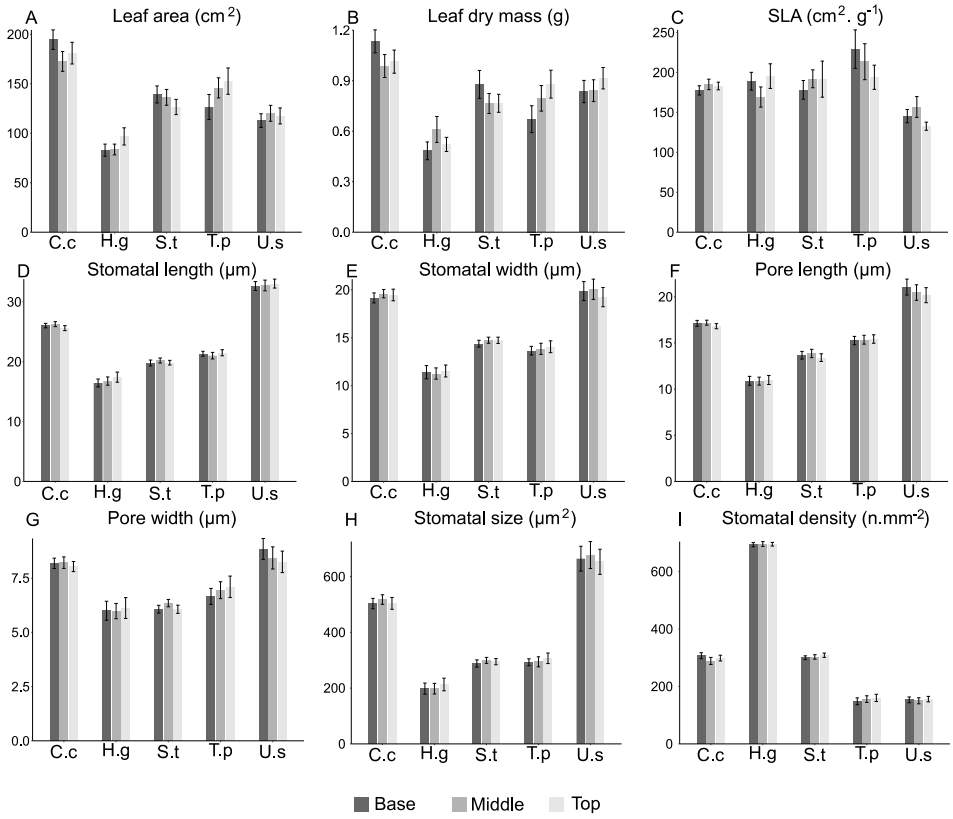

Figure 4-2. Variation in leaf traits as a function of position at different crown levels of the shrubs: the means of leaf area, dry mass, and specific leaf area of the different species studied. The thick bars are means, while the small bars above are standard error (SE). C.c = *Coffea canephora*, H.g = *Hua gabonii*, S.t = *Scaphopetalum thonneri*, T.p = *Tabernaemontana penduliflora*, U.s = *Uvariopsis solheidii*.

4.5.2. 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 4-3).

Table 4-3. 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

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 4-3; Fig. 4-3A). The leaf mass was not different between the two time periods, but there was a significant interaction effect with species (Table 4-3). 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 4-3; Fig. 4-3C). 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 4-3; Fig. 4-3D, E). In contrast, pore length did change over this period (Table 4-3; Fig. 4-3F). 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 4-3; Fig. 4-3E).

There was a significant interaction between collecting period and species for stomatal density (Table 4-3). 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. 4-3I). 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.

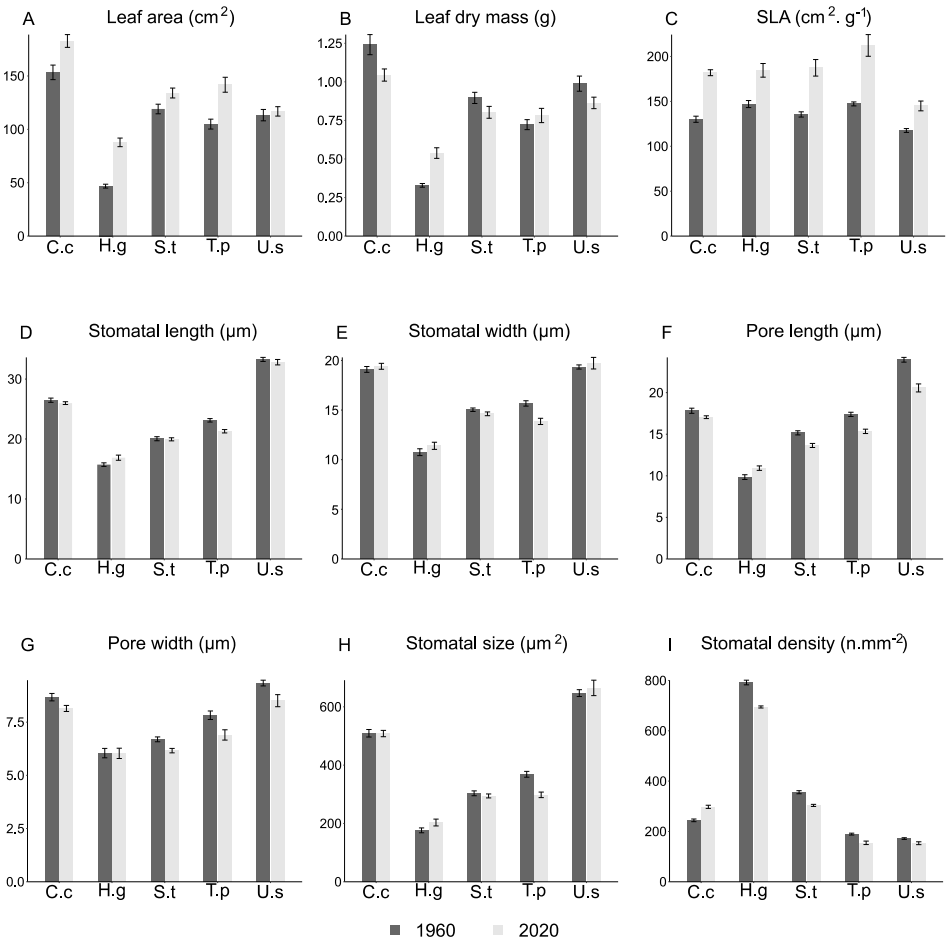

Figure 4-3. Variation of leaf traits of understory woody species between pre-1960 and 2019–2022. The bars represent average values, while the error bars are standard

errors (SE). C.c = *Coffea canephora*, H.g = *Hua gabonii*, S.t = *Scaphopetalum thonneri*, T.p = *Tabernaemontana penduliflora*, U.s = *Uvariopsis solheidii*.

4.6. 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.

4.6.1. 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).

4.6.2. 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 realize 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 standardized protocols (Pérez-Harguindeguy et al., 2013) is more reliable when using tropical forest understory species instead of canopy trees (Peñuelas and Matamala, 1990).

4.6.3. 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 global 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 Kaoru, 1996). Recordings from the meteorological institute at INERA Yangambi showed a rising temperature trend between 1961 and 2021 (Fig. 4-1A). 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 Kazadi and Kaoru (1996) who observed an increase in temperature throughout the Congo Basin, including the Yangambi region. In contrast to Kazadi and Kaoru (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 forest understory 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., 2009b; 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. (2015); 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).

4.7. 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 to build a solid database that can be used to draw more general conclusions about the future of tropical forests.

4.8. Acknowledgements

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4.9. Supplementary materials

4.9.1. Supplementary material 4-1

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

4.9.2. Supplementary material 4-2

Monthly average temperature and annual rainfall at the INERA Yangambi climatology station (DR Congo) between 1961 and 2021. Source: Yakusu et al. (2023).

Link: https://doi.org/10.5091/plecevo.104593.suppl2

Chapter 5. Spatiotemporal variation in *Coffea canephora* leaf traits and iWUE in Congo Basin forests

5.1. Background and rationale

The previous chapter highlighted the leaf physiology of understory woody species in the Congo Basin, including *C. canephora*, and the potential impacts of climate change over the past decades. Building on this, the present chapter focuses specifically on the responses of *C. canephora* to global environmental change, examining both morphological and physiological leaf traits. Understanding these responses is essential to evaluate the resilience of this economically and ecologically important understory species under ongoing climate change.

We employed a diachronic approach, combining leaf analyses from herbarium 'old' specimens collected between 1900 and 1960 with 'recent' collections made between 2016 and 2021 across multiple sites in the DRC. A total of 179 specimens were analyzed to capture temporal and spatial variation. Leaf traits measured included specific leaf area (SLA), stomatal density, stomatal and pore size, and maximum diffusive stomatal conductance to CO_2. Intrinsic water use efficiency (iWUE) was estimated using $\delta^{13}C$ values from leaf cellulose, providing insight into long-term physiological responses to rising atmospheric CO_2.

This chapter addresses four main objectives. First, to determine whether leaf morphological traits and iWUE of *C. canephora* have changed significantly over the past century. Second, to evaluate whether these changes are consistent across different

sites within the Congo Basin. Third, to assess the extent to which variation in leaf morphology explains differences in iWUE. Finally, to explore whether spatial and temporal variation in leaf traits and iWUE can be related to environmental gradients, including temperature, precipitation, and atmospheric CO_2.

By focusing on an understudied component of tropical forests, the woody understory, this chapter fills a critical gap in the literature. The findings provide new evidence on the resilience, phenotypic plasticity, and sensitivity of *C. canephora* to environmental change, offering important insights into the physiological and ecological dynamics of tropical rainforest ecosystems under climate change.

The content of this chapter is based on the following publication:

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Minor adaptations have been made to comply with university style guidelines.

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ORIGINAL ARTICLE

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

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5.2. Abstract

• **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* Pierre ex A. Froehner, a widespread understory species from West Africa to the African rift (Uganda).

• **Methods** Using 179 herbarium samples collected during two periods (1900–1960 and 2016–2021), we measured the specific leaf area (SLA), the stomatal size (S), the stomatal pore size (SPS), the stomatal density (SD), and the 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 understory species.

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

5.3. Introduction

Human activities continue to lead to increased emissions of the mean annual global greenhouse gas (GHG), 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 about 280 ppm during the preindustrial period to more than 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., 2009a, b), with the tropical forest carbon sink already 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 understory 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 & Wright (2007), for example, showed that functional relationships of photosynthetic capacity vary among growth forms and depend on microclimatic conditions. Forest understory species are not only exposed to lower light intensities but also to higher CO_2 concentrations compared to canopy trees (Hubau et al., 2019; Damasceno et al., 2024). Woody understory 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 understory 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 understory species in the Congo basin rainforest, *Coffea canephora* Pierre ex A. Froehner, 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 understory 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 to temperate ecosystems (Visscher et al., 2022). Among traits, the 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 (Mathias & Thomas, 2021; Belmecheri et al., 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 & 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 CO_2 atmospheric (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 understory 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 intrinsic water use efficiency (iWUE) in tropical understory 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 the SLA, the stomatal size (S), the stomatal pore size (SPS), the stomatal density (SD), the maximum diffusive stomatal conductance to CO_2 (g_{cmax}), the 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 understory shrub *C. canephora*. This unique setup enabled us to address the following questions: (i) Have the leaf traits and iWUE of *C. canephora* changed significantly over the past century? (ii) Are these temporal changes consistent across different sites? (iii) Do changes in leaf morphological traits explain the variation in iWUE? (iv) 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 (i) SLA and iWUE have increased over time, while SD and g_{cmax} decreased; (ii) these changes are consistent between sites in forests of the Congo basin forests; (iii) variation in leaf morphology explains the difference in iWUE; and (iv) leaf morphological and physiological traits and iWUE of *C. canephora* relate to environmental variation throughout the Congo basin.

5.4. Materials and methods

5.4.1. Sampling design and leaves conservation

Data were collected from 179 *C. canephora* herbarium specimens collected in DRC and kept in the Herbarium of Meise Botanic Garden (BR) in Belgium (Supplementary Data Table S5-1). Our data set included 89 'old' herbarium specimens collected throughout the DR Congo between 1900 and 1960, and 90 'recent' herbarium samples collected in four provinces (Tshopo, Ituri, Sankuru, and Bas-Uéle) between 2016 and 2021 (Fig. 5-1). Very few samples were collected between 1960 and 2016, and these were therefore not included in the study. We analyzed the Climatic Research Unit (CRU) Time-Series (TS) version 4.09 dataset (Harris et al., 2020) for historical air temperature (Supplementary Data Fig. S5-1) and precipitation (Supplementary Data Fig. S5-2) using the ncdf4 package (v1.24; Pierce 2025) in R (v4.4.2; 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 m 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 understory of the wet forest area, *C. canephora* shrubs usually flower when 5 to 10 m high (H. Yves, pers. Obs.). Subsequently, all dried leaves were dried at room temperature for 48 hours to standardize tissue condition in all samples.

5.4.2. Leaf morphological traits measurements

For each specimen, three replicate leaves were used for measurements of leaf morphological traits. Stomatal density and stomatal size were obtained by making microscopic impressions of the abaxial side of all three leaves, using the ''*kollodium method*'' (Stojnić et al., 2015) by applying colorless nail varnish on both sides of the main vein. After 12 to 16 hours, the varnish was removed using adhesive tape and placed on microscope slides following Meeus et al. (2020). Using a digital microscope (VH-5000 Ver 1.5.1.1, Keyence Corporation, Osaka, Japan), four photomicrographs of 1,600 × 1,200 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 × 1,000 (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 μm^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 vapor (g_{wmax}, mol m^{-2} s^{-1}) was calculated according to Franks & Beerling (2009), as follows:

$$g_{wmax} = \frac{d}{v}.SD.a_{max}/(W + \frac{\pi}{2}\sqrt{\frac{a_{max}}{\pi}}) \quad [1]$$

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

$$g_{cmax} = g_{wmax}/1.6 \quad [2]$$

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

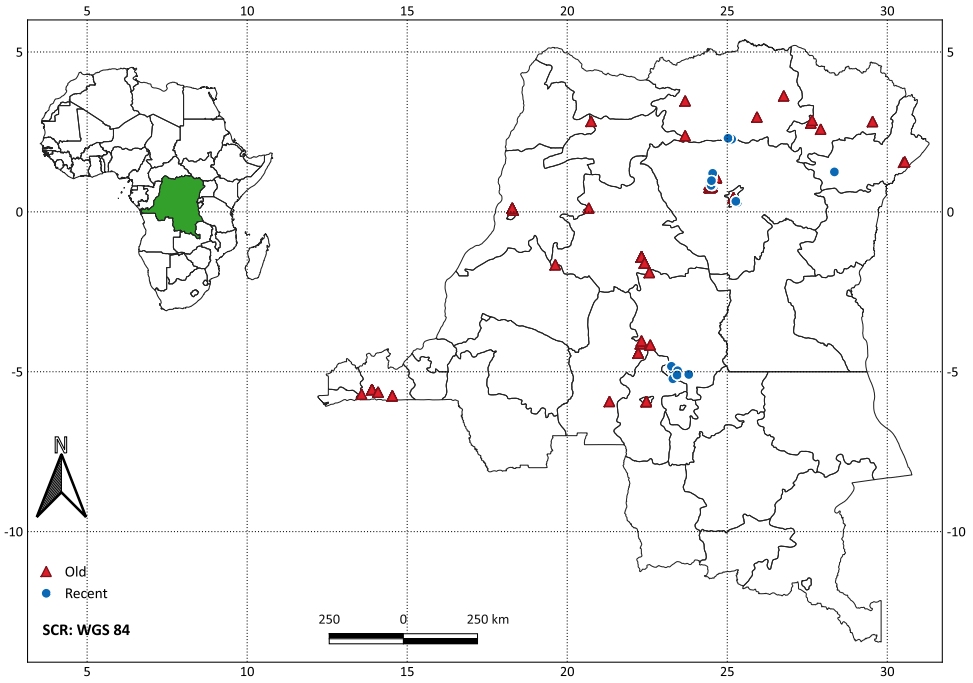

Figure 5-1. Location of old (red triangles) and recent (blue circles) herbarium samples collected in the DR Congo and used in this study. Old herbarium samples were collected between 1900 and 1960 in the understory of intact forests at the time of collection, but some of these forests have now disappeared. Recent samples have been collected between 2016 and 2021 in Congolese provinces where there are still intact forests today.

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 $y = 1.2x + -4.1$ ($R^2 = 0.97$) 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.

5.4.3. 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 & 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 SubAqua 34Plus 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 left for 1 hour. 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 minutes, then drained, followed by three Milli-Q rinses at room temperature. The samples were soaked in water for 12 hours to complete cellulose extraction. A second 45-minute 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-hour 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 hours to remove residual moisture. For $\delta^{13}C$ analysis, 0.8 ± 10 % mg of cellulose were weighed using an ultramicrobalance (RAWAG, 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). $\delta^{13}C$ 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 were weighed and placed in 6 × 4 mm silver capsules (Elemental Microanalysis, Okehampton, UK). The capsules were unsealed and oven dried for at least 5 days prior to measurement. The samples were analyzed using a high temperature thermal conversion element analyzer (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). $\delta^{18}O$ 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 $\delta^{13}C$–CO_2 ($\delta^{13}C_{atm}$) values from the equation in Bonal et al. (2011) calculation of the iWUE. We used the classic model of carbon isotope discrimination during CO_2 fixation in the leaves of C_3 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:

$$\Delta^{13}Cleaf = \frac{\delta^{13}C_{atm}\,(‰) - \delta^{13}C_{leaf}(‰)}{1 + \delta^{13}Cleaf(‰)/1000} \qquad [3]$$

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

$$C_i = \frac{C_{atm}\,(\Delta^{13}C_{leaf} - a) + f\Gamma*}{b - a} \qquad [4]$$

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 fΓ* 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).

Intrinsic water use efficiency (iWUE) is 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:

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

5.4.4. Environmental data acquisition

Using the geographical coordinates provided in the herbarium metadata (Supplementary Data Table S5-1), 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).

5.4.5. Data analysis

All analyzes were conducted in R (v4.4.2; R Core Team, 2025), with significance thresholds set at $p < 0.05$. Before statistical modeling, 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: 1900–1960; recent: 2016-2021), 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 (v3.4.8; 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). TS represents the variability of the temperature between years and was

calculated as the standard deviation of monthly mean temperatures x 100. PS reflects monthly rainfall variability and was expressed as the coefficient of variation (CV) of monthly precipitation totals, calculated as (SD of monthly precipitation / mean monthly precipitation) x 100. All models were fitted using maximum likelihood with the lmer() function from the lme4 package (v1.1-35.5; Bates et al., 2015b). The significance of fixed effects was evaluated using Wald's chi-square tests via the anova() function of the lmerTest package (v3.1.3; 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 (v1.48.4; Bartoń, 2024). The means and standard errors (SE) were grouped by region. All graphs were created using the ggplot2 package (v3.5.1; Wickham, 2016) and arranged using the plot_annotation() function from the patchwork package (v1.10.3; Pedersen, 2024).

5.5. Results

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

The leaf morphological traits of *C. canephora* exhibited significant long-term changes between 1900 and 2021. The specific leaf area (SLA) increased significantly over time (Table 5-1; $p<0.001$) 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. 5-2A). On the contrary, the stomatal density (SD) decreased significantly (Table 5-1; $p<0.001$) from 408.3 ± 12.2 mm^{-2} in 1900 to 317.2 ± 5.2 mm^{-2} in 2021 (-22%; Fig. 5-2D). No significant change in stomatal size (S) was observed over time (Fig. 5-2B; Table 5-1; $p>0.05$), which remained around 400 μm^2 on average. Stomatal pore size (SPS) decreased significantly over time (Table 5-1; $p<0.001$): pores were larger in 1900 (92.7 ± 2.8 μm^2) than in 2021 (78.3 ± 1.2 μm^2) (-16%; Fig. 5-2C).

The explanatory power of the models varied by trait (Table 5-1). 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, or the replicate of the herbarium. SD exhibited slightly lower explanatory power ($R^2_m = 0.06$; $R^2_c = 0.54$), S showed very low values ($R^2_m = 0.018$; $R^2_c = 0.48$), 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.

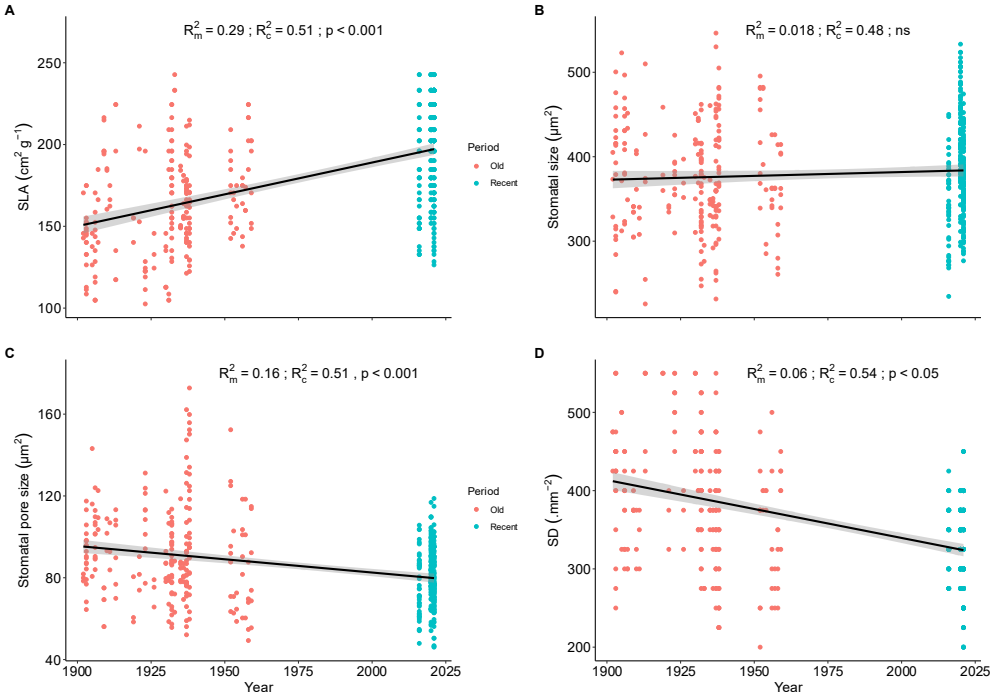

Figure 5-2. Temporal trends in *Coffea canephora* leaf morphological characteristics in forests of the Congo Basin based on linear regression models. The panels show results from linear regression models fitted across samples collected between 1900 and 2021. (A) Specific leaf area (SLA); (B) stomatal size; (C) stomatal pore size and (D) stomatal density. Each point represents an individual herbarium specimen, colored by collection period (red = old; blue = recent). The shaded areas indicate 95% confidence intervals around the fitted regression lines. Significance levels: ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001. R^2_c (conditional R^2): total variance explained by both fixed and random effects (including sampling region); R^2_m (marginal R^2) is variance explained by fixed effects.

The physiological characteristics of the leaves of *C. canephora* showed clear long-term trends over the past century (Table 5-1, Fig. 5-3). The $\delta^{13}C$ 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. 5A; $R^2_m = 0.53$, $R^2_c = 0.84$, $p < 0.001$). This trend is consistent with both the progressive depletion of atmospheric $\delta^{13}C$ and also indicates pronounced changes in the dynamics of carbon within the plant. In contrast,

$\delta^{18}O$ values remained relatively constant throughout the same period, with a mean of 29.7 ± 0.1‰ and no significant temporal trend (Fig. 5-3B; $R^2_m = 0.001$, $R^2_c = 0.03$, ns).

During the past century, a marked decline was observed in the maximum stomatal conductance to CO_2 (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. 5-3C; $R^2_m = 0.22$, $R^2_c = 0.53$, $p < 0.001$). The intercellular CO_2 concentration (C_i) increased substantially, from 228.0 ± 4.9 µmol mol^{-1} to 355.7 ± 4.5 µmol mol^{-1} (data not shown). These coordinated changes suggest a reduced stomatal conductance and increased CO_2 availability within the leaf, which could reflect long-term acclimatization to elevated atmospheric CO_2. Due to these changes, intrinsic water use efficiency (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. 5-3D; $R^2_m = 0.04$, $R^2_c = 0.67$, $p < 0.001$), indicating a reduced efficiency in balancing carbon assimilation against water loss under historical and contemporary environmental conditions. For isotopes and g_{cmax}, the marginal R^2 showed that Year explained a substantial part of the variance (0.36 for $\delta^{13}C$ and 0.16 for g_{cmax}; Table 5-1). The conditional R^2, 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, the 'year' explained a moderate amount of variance with a marginal R^2 of 0.00004, while the conditional R^2 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 S5-2.

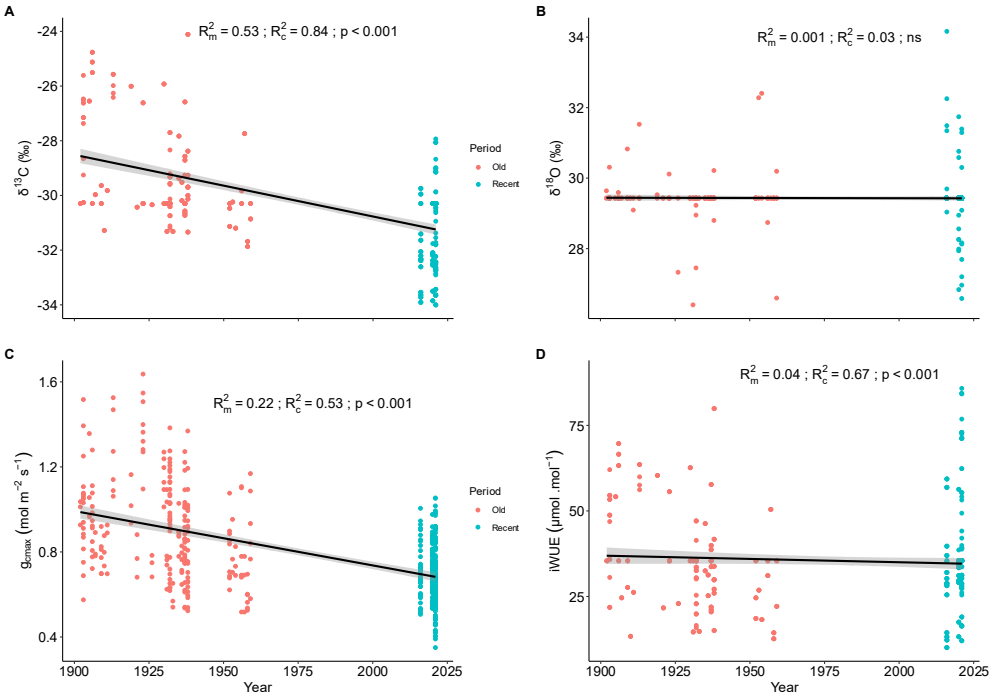

Figure 5-3. Temporal trends in the stable isotopic composition ($\delta^{13}C$, $\delta^{18}O$), maximum diffusive stomatal conductance to CO_2 (g_{cmax}) and intrinsic water use efficiency (iWUE) in *Coffea canephora* from forests in the Congo Basin. Panels show results from linear regression models 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, colored by collection period (red = old; blue = recent). Shaded areas indicate 95% confidence intervals around the fitted regression lines.

Table 5-1. Results of linear mixed effect models that examine the variation in leaf morphological and physiological traits, as well as intrinsic water use efficiency (iWUE), of *Coffea canephora* over time in Congo Basin forests. 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; $\delta^{13}C$: stable carbon isotope composition; $\delta^{18}O$: stable oxygen isotope composition; iWUE: intrinsic water use efficiency.

Traits	SLA	S	SPS	SD	g_{cmax}	$\delta^{13}C$	$\delta^{18}O$	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 ***	15.25 ns	51.44 ***	3.74 *	85.39 ***	39.73 ***	0.08 ns	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

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

The linear mixed effects models revealed both spatial and temporal variation of *C. canephora* leaf traits (Table 5-2, Fig. 5-4). 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. 5-4A) 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. 5-4B). 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. 5-4C). Stomatal density decreased significantly over time in the Kasaï and Uélé regions ($p<0.001$; Fig. 5-4D).

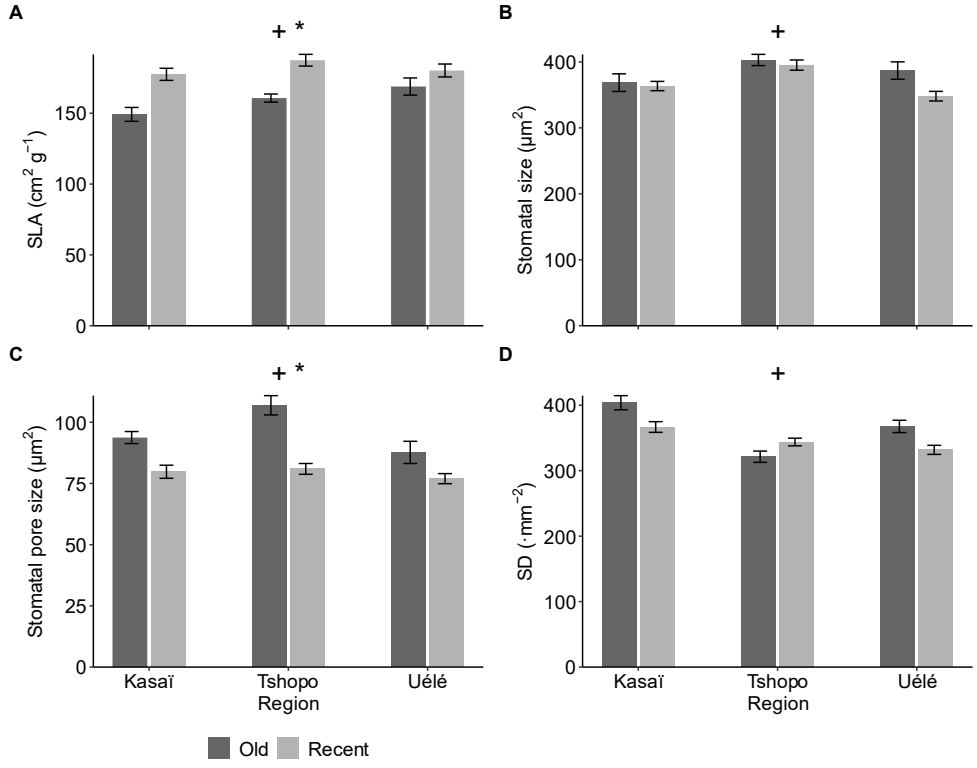

Figure 5-4. Temporal and spatial variation in the leaf morphological traits of *Coffea canephora* from three forest regions in the Congo basin. Bars represent means, and error bars denote standard errors (SE). Symbols indicate statistically significant effects based on linear mixed-effects models: + indicates a significant effect of region and * indicates a significant effect of collection period (old vs. recent).

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, stomatal density, g_{cmax}, $\delta^{13}C$ and C_i, while other traits showed no significant interactions (Table 5-2; $p < 0.001$). 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. 5-5A). In contrast, $\delta^{18}O$ values did not show significant variation between regions or over time (Table 5-2). 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 5-2; $p < 0.001$). From 1900 to

2021, C_i rose from 220.7 ± 4.2 µmol mol^{-1} to 343.1 ± 5.9 µmol mol^{-1} in Kasaï, from 253.0 ± 3.3 µmol mol^{-1} to 377.9 ± 2.8 µmol mol^{-1} in Tshopo, and from 265.4 ± 6.3 µmol mol^{-1} to 366.9 ± 3.9 µmol mol^{-1} in Uélé (Fig. 5-5B). The maximum diffusive stomatal conductance to CO_2 (g_{cmax}) also decreased significantly over time and varied between regions (Table 5-2; $p < 0.05$). A decrease from 1.1 ± 0.1 mol.m^{-2}. S^{-1} to 0.8 mol.m^{-2}. S^{-1} was observed in Kasaï, from 0.8 mol.m^{-2}. S^{-1} to 0.7 mol.m^{-2}. S^{-1} in Tshopo, and from 0.9 mol.m^{-2}. S^{-1} to 0.6 mol.m^{-2}. S^{-1} in Uélé (Fig. 5-5C). Finally, iWUE decreased over time and differed significantly between regions (Table 5-2; $p<0.001$). iWUE dropped from 49.2 ± 2.7 µmol mol^{-1} 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. 5-5D). More details on effects of sampling regions and collection period on *C. canephora* trait variation are given in Supplementary Table S5-3.

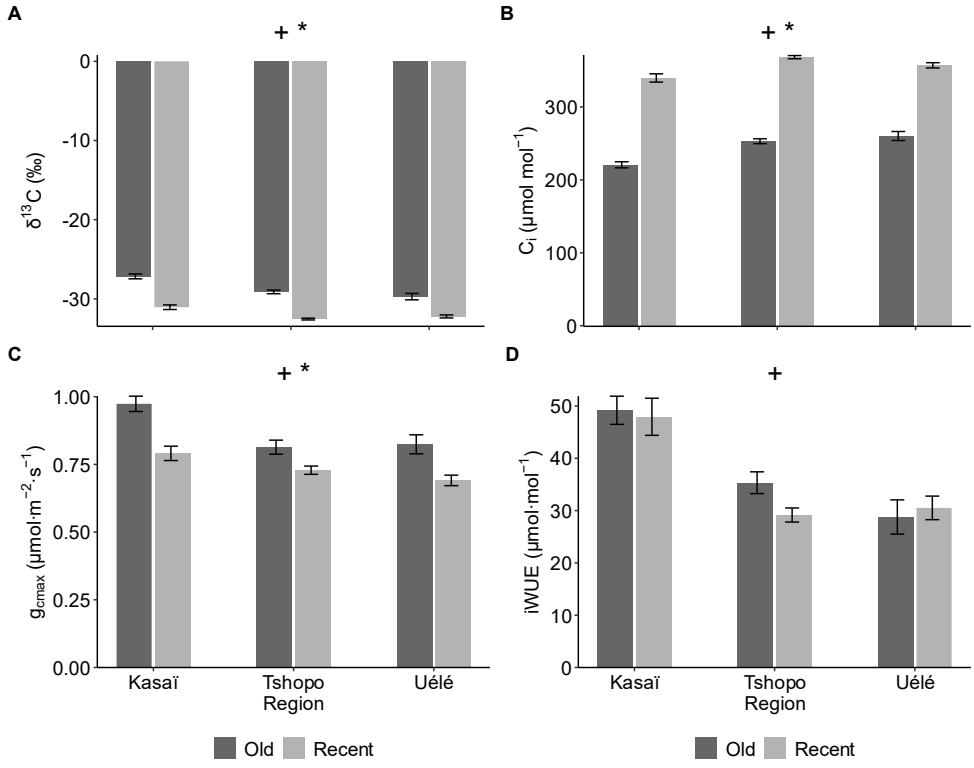

Figure 5-5. Temporal and spatial variation in the physiological traits of *Coffea canephora* leaves from three forest regions in the Congo Basin. Bars represent means, and error bars denote standard errors (SE). Symbols indicate statistically significant

effects based on linear mixed-effects models: + indicates a significant effect of region and * indicates a significant effect of collection period (old vs. recent).

Table 5-2. Summary of results of the linear mixed-effect models that analyze the effects of region and period on leaf morphological and physiological traits. 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. The significance levels are indicated as follows: ns = not significant; *p < 0.05; **p < 0.01; ***p < 0.001. Trait abbreviations: 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.

Traits	SLA	S	SPS	SD	g_{cmax}	$\Delta^{18}O$	$\Delta^{13}C$	C_i	iWUE
Fixed effects									
Intercept	5 ***	5.9 ***	4.48 ***	5.9 ***	-0.1 ns	3.4 ***	-27.22 ***	5.53 ***	3.96 ***
Region	7.38 ***	3.75 *	6.46 *	8.84 ***	7.83 ***	4.9 ns	21.32 ***	19.1 ***	23.32 ***
Period	3.34 *	0.86 ns	3.98 *	0.63 ns	4.11 *	2.54 ns	13.88 **	47.9 **	0.03 ns
Region* Period	1.11 **	0.62 ns	0.05 ns	8.98 **	4.65 *	0.05 ns	0.13 **	0.11* *	0.43 ns
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

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

Standardized Major Axis (SMA) regression revealed significant relationships between intrinsic water use efficiency (iWUE) and several key leaf traits. A strong negative relationship was observed between iWUE and specific leaf area (SLA) (Fig. 5-6A; $R^2 = 0.38$, $p < 0.001$), indicating that trees with higher SLA tend to have lower water use efficiency, highlighting a trade-off between leaf morphology and physiological performance. Furthermore, iWUE showed a moderate positive

association with stomatal density (Fig. 5-6C; $R^2 = 0.18$, $p < 0.001$), suggesting that a higher stomatal density contributes to increased water use efficiency. Stomatal size exhibited a weaker but significant positive relationship with iWUE (Fig. 5-6B; $R^2 = 0.043$, $p < 0.05$), indicating that larger stomata may also slightly improve water use efficiency. Furthermore, the maximum stomatal conductance (g_{cmax}) showed a moderate positive correlation with iWUE (Fig. 5-6D; $R^2 = 0.24$, $p < 0.001$), 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. S5-3.

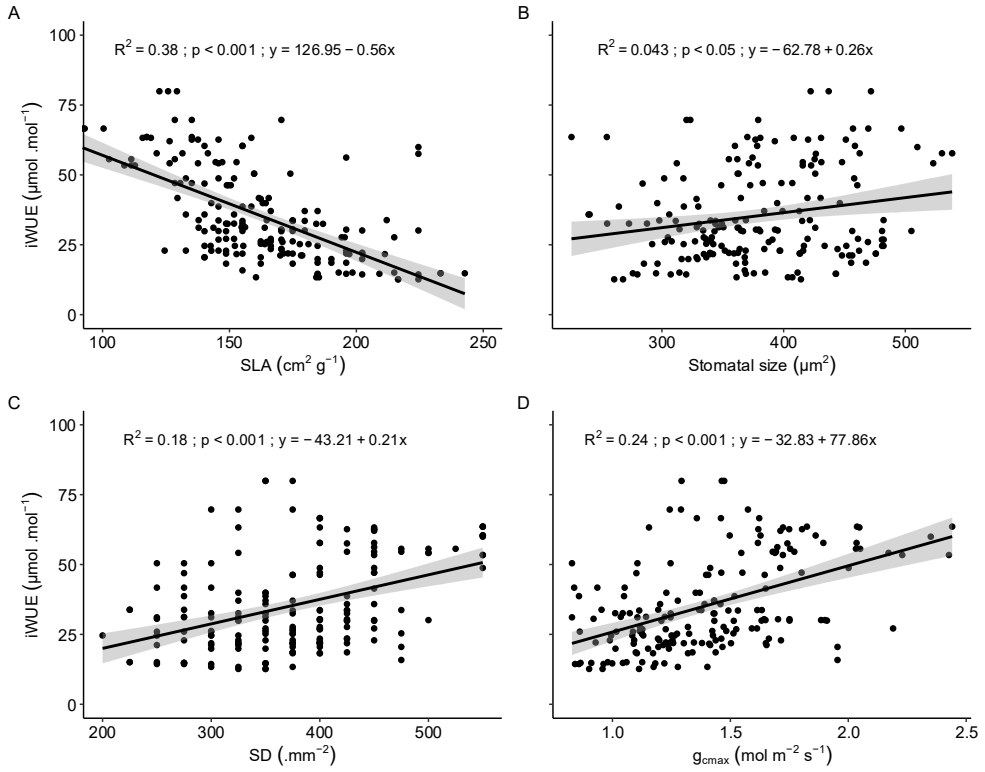

Figure 5-6. Relationships between leaf morphological traits and intrinsic water use efficiency (iWUE) in *Coffea canephora* from forests of the Congo Basin, based on 'old' herbarium samples collected between 1900 and 1960. Each panel shows a linear regression between iWUE and a morphological trait: (A) 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.

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

Environmental and geographic variables significantly influenced key leaf traits of *C. canephora* throughout the Congo Basin (Table 5-3). 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 the pore size also showing a marginal positive effect of MAP ($F = 3.15$, $p = 0.082$). The 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: the maximum stomatal conductance (g_{cmax}) decreased significantly with C_{atm} ($F = 4.70$, $p = 0.043$); internal CO_2 concentration (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, temperature seasonality, and precipitation seasonality generally had no significant effect on physiological traits. Marginal R^2 values ranged from 0.08 to 0.46, and conditional R^2 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.

Table 5-3. Fixed-effect coefficient estimates from linear mixed-effect models that examine the influence of environmental variables on leaf morphological and physiological traits and iWUE of *C. canephora*. Abbreviations: 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, iWUE: intrinsic water use efficiency. 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). The numbers are the coefficients of the LMM. Only statistically significant coefficients ($p < 0.05$) are indicated with an asterisk (*). The significance levels are indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Marginal (R^2_m) and conditional (R^2_c) R-squared values represent the variance explained by fixed effects alone and by fixed and random effects, respectively.

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

5.6. 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 understory of the Congo Basin rainforest. We observed an overall increase in SLA of *C. canephora* shrubs over the past century, while stomatal density 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 atmospheric CO_2 concentration (C_{atm}) (Medlyn *et al.*, 2011; Gardner *et al.,* 2023), we observed a decline in iWUE that was not driven by increasing stomatal conductance. Instead, the stomatal conductance also declined, suggesting that photosynthetic capacity may have diminished over time.

5.6.1. 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 understory woody species, one of which was also *C. canephora*, in Yangambi, DR Congo, 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., 2009a, b; Hubau et al., 2020; Wang et al., 2024). Although understory species generally have a low photosynthetic CO_2 assimilation rate per area and a high SLA compared to canopy trees (da Silveira et al., 1989; Santiago & Wright, 2007), they do respond to atmospheric CO_2 enrichment by adjusting their main functional traits, especially their stomatal behavior from intracellular signaling to whole-plant responses to global climatic change (Woodward, 1987; Woodward et al., 2002; Hetherington & 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 2020-2021. These more negative $\delta^{13}C$ values are consistent with the physiological responses expected in understory species growing under low light

conditions, where limitations in photosynthetic capacity lead to a decrease in intrinsic water-use efficiency (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 understory and shaded 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 understory 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 understory 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/C_a, reflecting greater isotopic discrimination during photosynthesis and resulting from either by increased stomatal conductance or reduced 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, stomatal size, and iWUE over the past century, along with a decrease in stomatal density and stomatal conductance. Our results only partially support this hypothesis: while SLA increased and both stomatal density and conductance 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).

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 stomatal conductance), 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. (2024), who mentioned stomatal conductance and photosynthesis decreased with warming. Furthermore, it is known through previous studies that iWUE increases proportionally with an increase in 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 & 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 stomatal size, a decrease in stomatal density, 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 stomatal density and smaller stomatal size (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).

5.6.2. Environmental drivers of leaf traits and iWUE

We observed a strong negative relationship between intrinsic water use efficiency (iWUE) and specific leaf area (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 between iWUE and stomatal size, stomatal density, and maximum stomatal conductance, consistent with previous studies. In particular, our results confirm the strong positive relationship between iWUE and stomatal density 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 stomatal density 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: stomatal density decreased, whereas stomatal size increased in response to long-term elevated [CO_2]. Our findings suggest that the relationship between stomatal density and iWUE is context dependent, shaped by environmental conditions, species-specific physiology, and growth strategy. A higher stomatal density 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 stomatal density, evidence from other systems shows that lower stomatal density can also improve water use efficiency. For instance, Petrík

et al. (2023) reported that reduced stomatal density enhances iWUE by limiting transportational water loss; similar findings were published by Stojnić et al. (2019) for one-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 favorable 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 & 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 stomatal conductance, 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 favorable 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 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.

5.6.3. 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 specific leaf area (SLA), stomatal size, and stomatal pore size, were more strongly shaped by spatial climatic gradients, especially mean annual temperature (MAT) and latitude. On the contrary, physiological traits such as maximum stomatal conductance (g_{cmax}) and intercellular CO_2 concentration (C_i) exhibited a greater sensitivity to atmospheric CO_2 concentrations (C_{atm}) and mean annual precipitation (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 a physiological traits variation 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 favorable thermal and light conditions, positively correlated with photosynthetic traits (Wright et al., 2005; Poorter et al., 2009 ; Li & 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 covariate with temperature and irradiance along latitudinal gradients (Hetherington & Woodward, 2003; Milla, 2023). Although stomatal density 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 behavior 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 stomatal conductance 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 & 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 and/or plasticity 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., 2010a) and expand trait-based analyses to include reproductive and belowground traits for a more comprehensive understanding of species-level adaptation strategies in both cultivated and wild populations.

5.7. Conclusions

Our study provides strong evidence that *C. canephora*, a key understory 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 specific leaf area (SLA), stomatal size (S), and intercellular CO_2 concentration (C_i) have been noted. In parallel, the decreases in stomatal density, stomatal pore size, maximum diffusive stomatal conductance to CO_2 (g_{cmax}), $\delta^{13}C$, and intrinsic water use efficiency (iWUE), indicating a shift toward 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, challenging the expectations from stomatal optimization theory have been observed. 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 understory species and integrate physiological measurements with demographic and reproductive traits to refine predictions of species resilience under ongoing climate change.

5.8. Supplementary data

Supplementary data are available at Annals of Botany online and consist of the following: Table S5-1: data representing the morphological and physiological traits of the 179 *Coffea canephora* herbarium specimens analyzed in this article. Table S5-2: results of LMMs testing the effects of year, region and their interaction on leaf functional traits of *Coffea canephora*. Table S5-3: results of LMMs testing the effects of region, period and their interaction on foliar traits of *Coffea canephora*. Figure S5-1: temporal trends in mean annual temperature at the main sampling sites from 1901 to 2024. Figure S5-2: Temporal trends in annual precipitation at the main sampling sites from 1901 to 2024. Figure S5-3: 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 S5-1 that supports the findings of this study is available on Zenodo at https://zenodo.org/ records/17176173.

5.9. Funding

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5.11. Conflicts of interest

None declared.

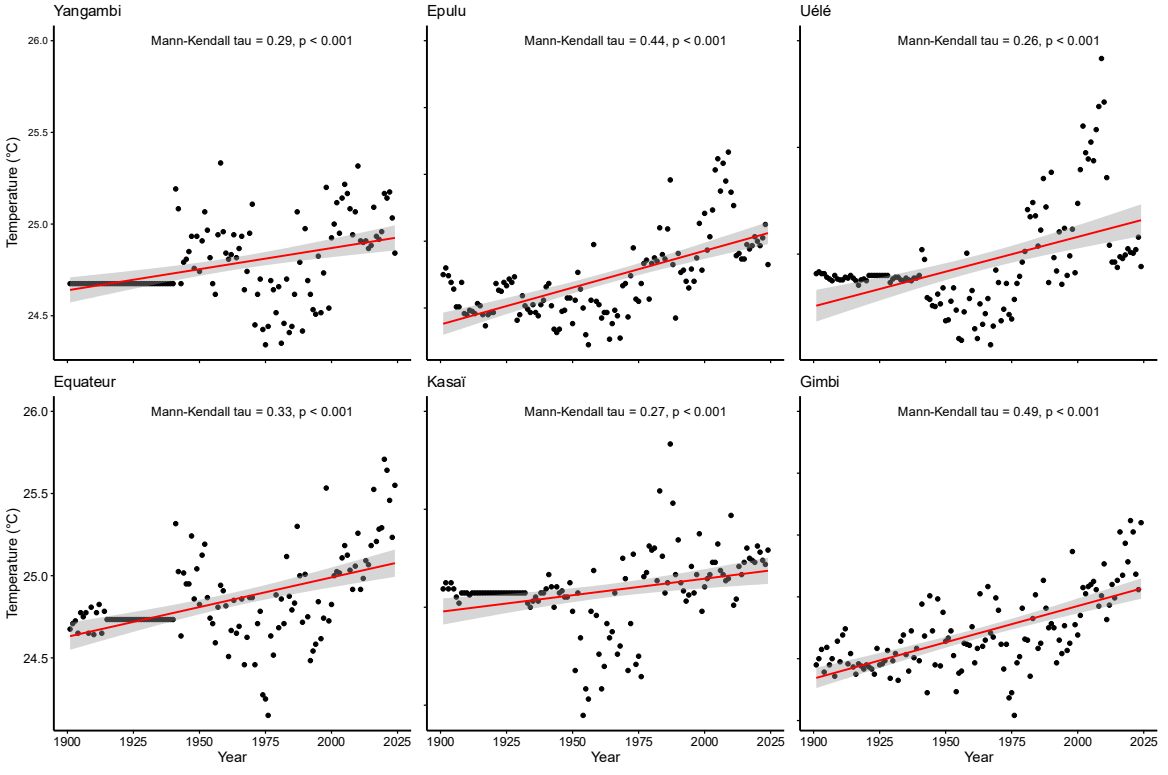
5.12. Supplementary information

5.12.1. Supplementary methods

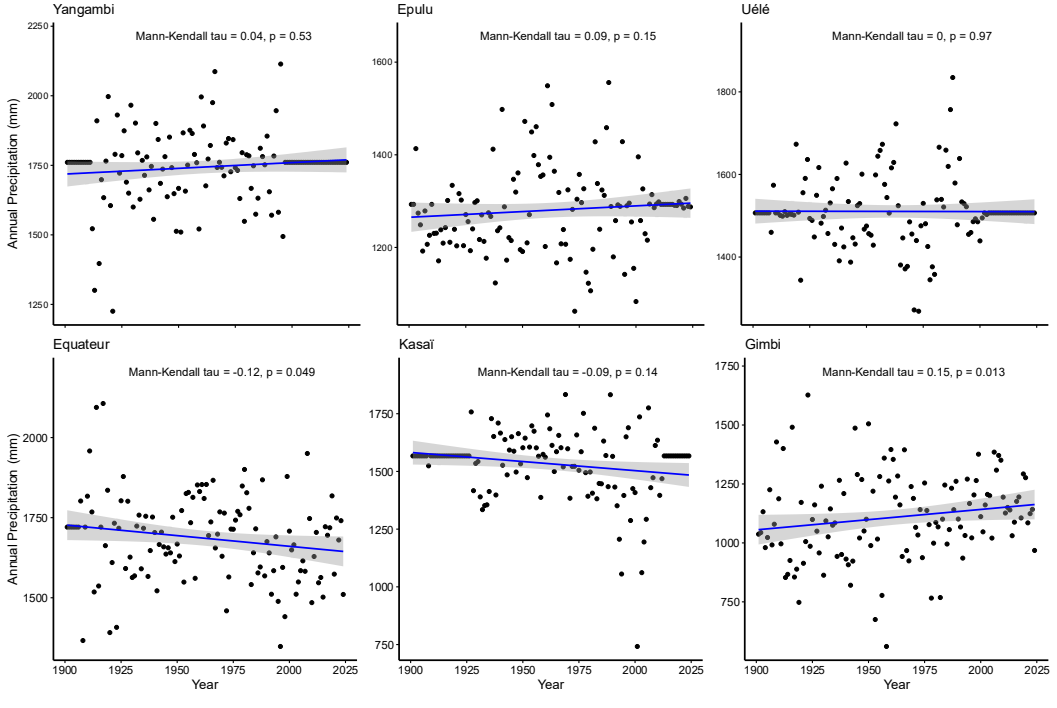
Supplementary Table S5-1. Data used in the preparation of the manuscript represent the morphological and physiological traits of the 179 *Coffea canephora* herbarium

specimens analyzed to produce the results presented in the article. The data are available on Zenodo at https://zenodo.org/records/17176173.

To characterize the main sampling sites, we downloaded historical climate data from the Climatic Research Unit (CRU) Time-Series (TS) version 4.09 dataset, which is publicly accessible at https://data.ceda.ac.uk/badc/cru/data/cru_ts/cru_ts_4.09/data/ (Harris et al., 2020). This dataset provides monthly gridded information on air temperature and precipitation from 1901 to 2024. NetCDF files were imported into R using the ncdf4 package (v1.24; Pierce 2025), with the nc_open() and ncvar_get() functions to read coordinates and climate variables. Variables corresponding to each sampling site were extracted by identifying the nearest grid cell with which.min(). Monthly data were aggregated to annual means for temperature and total precipitation using dplyr (v.1.1.4; Wickham *et al.* 2023) functions (mutate(), group_by(), summarize()) and formatted into tidy data frames with tibble(). Missing values were checked and handled according to CRU guidelines. Time-series plots were generated using ggplot2 (v3.5.1; Wickham, 2016), with geom_point() for annual values, geom_smooth(method = "lm") for linear trends, and annotate() to display Mann-Kendall trend test results MannKendall() from the Kendall package (v.2.2.1; McLeod 2022). Additional data wrangling was performed with tidyr functions as needed, and plots were customized for clarity and comparability across sites (Figures S5-1 & S5-2).



Supplementary Figure S5-1. Temporal trends in mean annual temperature at the main sampling sites from 1901 to 2024. Data source: Centre for Environmental Data Analysis (CEDA), CRU TS v4.09 dataset.



Supplementary Figure S5-2. Temporal trends in annual precipitation at the main sampling sites from 1901 to 2024. Data source: Centre for Environmental Data Analysis (CEDA), CRU TS v4.09 dataset.

5.12.2. Supplementary results

Linear mixed-effects models revealed significant effects of both Year and Region on several leaf traits of *Coffea arabica* (Supplementary Table S5-2).

Supplementary Table S5-2. Results of linear mixed-effects models testing the effects of Year, Region, and their interaction on leaf functional traits of *Coffea canephora* in the Congo Basin forests. Reported are fixed-effect coefficients, F-values, and model fit statistics. Significance levels: ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Marginal (R^2m) and conditional (R^2c) coefficients of determination are shown for each model. Trait abbreviations: 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.

Traits	SLA	S	SPS	SD	g_{cmax}	$\delta^{13}C$	$\delta^{18}O$	iWUE
Intercept	2.74	19.07***	11.06**	7.54*	4.39	118.04***	213.28.	44.73***
Coefficient								
Year	0.0012	-0.01***	-0.0035	-0.0008	-0.002	-0.08***	-0.10.	-0.02***
Kasaï	-1.76	-14.06***	-3.65	2.13	1.11	-77.79*	-142.96	-38.79***
Tshopo	-2.74	-13.19***	-2.24	-1.88	-1.69	-72.84*	-206.4.	-38.73***
Uélé	-0.47	-11.58***	-2.4	2.58	2.2	-97.08**	-149.16	-44.68***
F-value								
Year	30.71***	15.53***	13.36***	7.40**	17.88***	126.21***	6.32*	33.69***
Region	3.21*	6.57***	0.51ns	6.72**	2.68*	9.19***	1.69ns	9.76***
Year*Region	3.23*	6.68***	0.49ns	6.41**	2.66*	9.07***	1.68ns	9.66***
Model Fit								
R^2_m	0.34	0.23	0.18	0.33	0.35	0.55	0.31	0.25
R^2_c	0.47	0.42	0.32	0.59	0.55	0.83	0.88	0.7

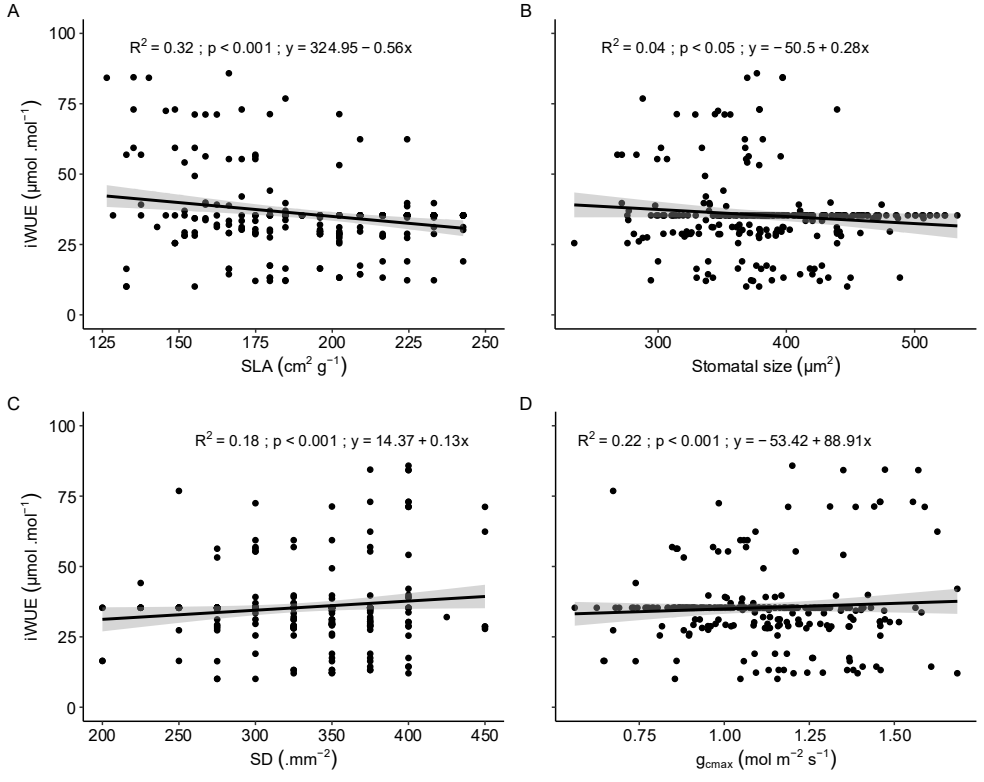
Year exerted a strong influence on specific leaf area (SLA; $F = 30.71$, $p < 0.001$), stomatal size ($F = 15.53$, $p < 0.001$), stomatal pore size ($F = 13.36$, $p < 0.001$), stomatal density ($F = 7.40$, $p < 0.01$), maximum stomatal conductance ($F = 17.88$, $p < 0.001$), leaf carbon isotope composition ($\delta^{13}C$; $F = 126.21$, $p < 0.001$), leaf oxygen isotope composition ($\delta^{18}O$; $F = 6.32$, $p < 0.05$), and intrinsic water-use efficiency ($F = 33.69$, $p < 0.001$). Regional differences were also detected, particularly for stomatal size ($F = 6.57$, $p < 0.001$), stomatal density ($F = 6.72$, $p < 0.01$), maximum stomatal conductance ($F = 2.68$, $p < 0.05$), $\delta^{13}C$ ($F = 9.19$, $p < 0.001$), and iWUE ($F = 9.76$, $p < 0.01$). A significant interaction between Year and Region was observed for SLA ($F = 3.23$, $p < 0.05$), stomatal size ($F = 6.68$, $p < 0.001$), stomatal density ($F = 6.41$, $p < 0.01$), maximum stomatal conductance ($F = 2.66$, $p < 0.05$), $\delta^{13}C$ ($F = 9.07$, $p < 0.001$), and iWUE ($F = 9.66$, $p < 0.01$). These results indicate that temporal changes in trait values varied between regions. The models explained a substantial proportion of the variance in trait values, with marginal R^2 values ranging from 0.18 (SPS) to 0.55 ($\delta^{13}C$), and conditional R^2 values ranging from 0.32 (SPS) to 0.88 ($\delta^{18}O$).

Mixed-effect models tested the effects of Region Period, and their interaction on foliar morphological and isotopic traits of *Coffea canephora* (Table S5-3).

Supplementary Table S5-3. Results of linear mixed-effects models testing the effects of Region, Period, and their interaction on foliar traits of *Coffea canephora*. Shown are F-values (with significance levels: p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***, ns = not significant) and parameter estimates for each factor level. Model fit is reported as marginal R^2 (R^2_m; variance explained by fixed effects) and conditional R^2 (R^2_c; variance explained by both fixed and random effects). Trait abbreviations: 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; $\delta^{18}O$, stable oxygen isotope; iWUE, intrinsic water use efficiency.

Traits	**SLA**	**S**	**SPS**	**SD**	**g_{cmax}**	**$\delta^{18}O$**	**$\delta^{13}C$**	**C_i**	**iWUE**
Intercept	5.03	5.88	4.53	5.98	-0.05	-27.21	3.41	5.39	3.77
Estimate									
Tshopo	0.009	0.1	0.08	-0.238	-0.207	-1.93	-0.0005	0.14	-0.2
Uélé	0.13	0.04	-0.09	-0.07	-0.161	-3.69	0.004	0.21	-0.86
Period	3.34	0.86	3.98	0.63	4.11	-2.83	-0.001	0.36	0.27ns
Tshopo * Recent	0.09	-0.03	0.004	0.24	0.25	-0.06	-0.001	-0.03	-0.32
Uélé * Recent	-0.04	-0.09	0.04	-0.14	-0.1	0.24	0.001	-0.03	-0.23ns
Region F-value	1.77***	3.69*	2.72*	4.18***	4.43*	14.34ns	2.75***	14.04***	9.79***
Period F-value	2.22*	0.41ns	4.73*	0.18*	3.15ns	11.92ns	0.45**	47.99**	0.09ns
Region * Period F	1.11**	0.62ns	0.05ns	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

Interactions between sampling regions and collection periods were significant for SLA, stomatal density, g_{cmax}, $\delta^{13}C$ and C_i, while other traits showed no significant interactions. Marginal R^2 (R^2_m) ranged from 0.05 (S) to 0.70 (C_i), while conditional R^2 (R^2_c) was consistently high, from 0.48 (S) to 0.97(iWUE), indicating strong contributions of random effects (Year and individual plants) in explaining trait variation.



Supplementary Figure S5-3. Relationships between leaf morphological traits and intrinsic water use efficiency (iWUE) in *Coffea canephora* from forests of the Congo Basin, based on 'recent' herbarium samples collected between 2016 and 2021.

Chapter 6. Phenotypic plasticity of growth and functional traits in *Coffea canephora* seedlings along an altitudinal gradient in the Congo Basin

6.1. Background and rationale

The previous chapter examined long-term changes in leaf functional traits and intrinsic water-use efficiency of *C. canephora* in the Congo Basin, highlighting the species' physiological responses to historical environmental change. This chapter examines its contemporary performance along an altitudinal gradient, with particular emphasis on phenotypic plasticity. It aims to assess how growth, leaf morphology, and stomatal traits vary among genetic origins and sites characterized by contrasting environmental conditions, thereby elucidating the role of phenotypic plasticity in shaping plant responses to environmental heterogeneity.

To address this objective, we conducted a multi-location trial using three *C. canephora* varieties, established at three sites spanning low (450 m), mid (800 m), and high (1,700 m) elevations in the Congo Basin. This experimental design allows the simultaneous evaluation of environmental and genetic effects on *C. canephora* seedling growth and functional trait expression. Growth traits, leaf morphology, and stomatal characteristics were measured over one year, alongside detailed climatic and soil assessments at each site.

Given projected warming in tropical regions, understanding altitudinal responses is critical. Although *C. canephora* is more thermotolerant than *Coffea arabica*, its performance at higher elevations remains poorly documented. Evaluating diverse genetic resources is therefore essential for climate-resilient coffee production.

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Minor adaptations have been made to comply with university style guidelines.

Phenotypic plasticity of growth and functional traits in *Coffea canephora* seedlings along an altitudinal gradient in the Congo Basin

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6.2. Abstract

Understanding how perennial crops adapt to environmental heterogeneity is critical for maintaining productivity under increasing climatic variability in tropical regions. We evaluated growth and functional performance of three genetically distinct varieties (origins) across sites located along an altitudinal gradient ranging from 450 to 1,700 m a.s.l. Seedlings from three origins were cultivated for one year at three sites representing low, mid, and high elevations (450, 800 and 1,700 m a.s.l.): Yangambi, Mambasa, and Mulungu, respectively. We measured eight morphological, growth, and functional traits and four stomatal traits. Trait variation and site-origin interactions were analyzed using linear mixed-effects models, permutation test for homogeneity of multivariate dispersions, and principal component analysis (PCA).

Seedling performance varied strongly among sites and was consistently influenced by varietal origin. Traits associated with elongation growth and leaf expansion, including plant height, leaf number, internode length, and specific leaf area, decreased toward higher-elevation sites, indicating reduced elongation growth and leaf expansion under cooler conditions. In contrast, traits associated with structural investment and biomass accumulation, including final stem diameter and above-ground biomass, increased at higher altitude. Stomatal traits also varied across the gradient, with changes in stomatal size and density, and pore size, suggesting adjustments in gas-exchange strategies. Multivariate analyses revealed clear phenotypic differentiation among site-origin combinations.

Our findings demonstrate pronounced structuring of growth and functional traits across sites along the altitudinal gradient, highlighting the importance of genotype-specific responses and phenotypic plasticity in shaping seedling performance under heterogeneous tropical environments. These results provide valuable insights for selecting varieties suited to specific agroecological conditions and for anticipating crop responses under future climate scenarios. Findings also provide initial evidence that Congolese *C. canephora* genotypes can establish at elevations up to 1700 m, although more research is warranted to assess long-term productivity.

Keywords

Altitudinal gradient, Coffee production, local adaptation, morphological traits, stomatal traits, tropical agroecosystems.

6.3. Introduction

Climate change is altering the atmospheric and hydrological drivers that regulate plant carbon assimilation, water use, and growth, with major consequences for agricultural systems. Rising temperatures, modified precipitation regimes, and more frequent climate extremes represent the dominant signals of recent change (IPCC, 2023). At the crop scale, these trends translate into higher leaf and canopy temperatures, altered radiation environments, and increased atmospheric evaporative demand, commonly expressed as vapor pressure deficit (VPD). Elevated VPD increases transpirational demand and frequently constrains stomatal conductance, thereby coupling canopy energy balance with reduced CO_2 uptake and growth, particularly when soil water availability is limited (Grossiord et al., 2020; Novick et al., 2024). In managed perennial systems, these coupled heat–VPD–soil moisture interactions can reshape productivity and stability over decadal timescales.

Tropical agroecosystems are particularly exposed because many crops already operate near their upper thermal limits (Sentinella et al., 2020). In these regions, warming often coincides with higher evaporative demand, more frequent droughts, and increasingly erratic rainfall, resulting in documented yield declines across tropical climates (Raju et al., 2024; Tran et al., 2025). Perennial crops are especially sensitive due to long life cycles, fixed plantation locations, and narrow phenological requirements, which constrain rapid varietal turnover or spatial relocation (Challinor et al., 2014). Coffee exemplifies this vulnerability: its phenology, yield formation, and bean quality are tightly regulated by temperature and water availability and are strongly mediated by microclimatic conditions (DaMatta et al., 2018; Tournebize et al., 2022).

Global mean temperature has increased by approximately 1 °C since pre-industrial times, with projections indicating a further rise of 1.5-6 °C by 2100 depending on emission trajectories (Agbo and Edet, 2022; IPCC, 2023; Forster et al., 2025). Together with altered precipitation regimes, this warming is expected to reduce the climatic suitability of many traditional coffee-growing regions, particularly at lower elevations of Robusta's areal (<500 m a.s.l.) (Läderach et al., 2017; Semedo et al., 2018). Elevated canopy temperatures, higher VPD, and increased radiation loads intensify leaf-level heat and water stress, with direct consequences for stomatal regulation, photosynthetic carbon assimilation, and reproductive success (DaMatta et al., 2018). Consistent with these ecophysiological constraints, climate-impact modelling studies predict substantial reductions in areas suitable for *Coffea arabica* (trade as Arabica) cultivation by mid-century (Davis et al., 2012; Bunn et al., 2015;

Moat et al., 2017; DaMatta et al., 2018; Tournebize et al., 2022). In addition, wild populations of *C. arabica* may face severe extinction risks in their native ranges in Ethopia and South Sudan, further threatening genetic resilience (Davis et al., 2019; Moat et al., 2018).

Recent CMIP6-based assessments further highlight increasing compound risks from the co-occurrence of heat and water stress in major producing regions (IPCC, 2023; Dias et al., 2024). Modelling studies consistently identify temperature, rainfall, and atmospheric humidity as key determinants of coffee suitability and yield (Davis et al., 2012; Tournebize et al., 2022; Adane, 2024; Leiva et al., 2024). Production zones between 500 and 800 m a.s.l. are expected to experience particularly strong impacts as temperatures increasingly exceed the thermal tolerance of *C. arabica*, while mid- and higher altitudinal belts (800–1200 m) are also projected to face growing climatic stress (Läderach et al., 2017; Campuzano-Duque et al., 2021). To maintain coffee production, two broad adaptation pathways are therefore widely discussed: modification of farming systems to buffer crops against adverse microclimatic conditions (e.g. agroforestry), and diversification toward more stress-resilient Coffea species and cultivars. *Coffea canephora* is frequently proposed as a promising alternative due to its comparatively higher tolerance to several biotic and abiotic stresses and its broad genetic diversity, particularly within wild populations (Davis et al., 2021; Kiwuka et al., 2021; Vanden Abeele et al., 2021; Depecker et al., 2023; Ferrão et al., 2024). In mid-elevation environments, sustaining productivity will likely require a combination of species diversification and the deployment of improved genotypes with enhanced tolerance to heat and drought.

Coffee species ecophysiological studies provide mechanistic insight into the limits and tradeoffs underlying these adaptation options. Under combined drought and high-temperature stress, both *C. arabica* and *C. canephora* enhance photoprotective and antioxidant defenses and impose tighter stomatal regulation, thereby limiting oxidative damage and maintaining photosystem II efficiency (Dubberstein et al., 2020; Rodrigues et al., 2024). Nevertheless, *C. arabica* exhibits a sharp decline in photosynthetic performance at leaf temperatures above 37–39 °C, indicating limited acclimation capacity beyond this threshold (Martins et al., 2016; Rodrigues et al., 2016, 2024). Although *C. canephora* generally shows greater thermotolerance, it remains sensitive to prolonged water deficits, indicating that no single species provides a complete solution to intensifying climate stress (Kiwuka et al., 2023). These biophysical vulnerabilities have important socio-economic implications. Coffee is among the most valuable traded agricultural commodities globally and is produced predominantly by smallholder farmers in tropical regions, making the sector

highly sensitive to climate-driven yield instability (ICO, 2024). Globally, more than 125 million people depend directly or indirectly on coffee for their livelihoods (IISD, 2022), while global supply relies almost exclusively on *C. arabica* and *C. canephora*, amplifying system-level exposure to climate change (ICO, 2024).

Altitudinal gradients provide natural laboratories to study plant responses to environmental variation, as they integrate multiple environmental factors, including changes in temperature, atmospheric pressure, radiation, and soil characteristics (Körner, 2007). They are particularly suitable for investigating phenotypic plasticity because they encompass strong, spatially structured environmental variation that elicits plastic responses in plant morphology, physiology, and phenology (Nicotra et al., 2010b; Sommer, 2020; Chen et al., 2024). In parallel, such gradients also allow the assessment of potential signals of local adaptation among populations. Phenotypic plasticity refers to the capacity of a genotype to express different phenotypes across environments, providing an immediate buffering mechanism against environmental variability (Bradshaw, 1965; Nicotra et al., 2010b; Stotz et al., 2021; Laitinen, 2024). In contrast, local adaptation describes heritable genetic differentiation among populations that results in higher fitness in their native environments and often leads to trait divergence along environmental gradients (Valladares et al., 2014; Liu et al., 2020). For coffee cultivation, altitudinal shifts are increasingly considered as a potential strategy to buffer rising temperatures. Zones currently cultivated with Arabica at mid-elevations may become suitable for Robusta under future climate scenarios (Läderach et al., 2017; Campuzano-Duque et al., 2021). Yet, empirical data evaluating the growth performance and functional responses of *C. canephora* genotypes along broad altitudinal gradients remain limited, particularly in its native Congo Basin range.

Assessing the adaptive potential of *C. canephora* therefore requires evaluation of its performance across environmental gradients representative of current and future coffee-growing regions. While *C. canephora* is often reported to outperform *C. arabica* under high temperatures and water limitation (Bunn et al., 2015; Ferrão et al., 2024), this assumption has been challenged by evidence of lower optimal growth temperatures and strong genotype-by-environment interactions (Kath et al., 2020). Furthermore, Robusta cultivation currently relies on a relatively narrow genetic base, increasing vulnerability to environmental stressors (DaMatta et al., 2018; Davis et al., 2020). Incorporating wild genetic resources into experimental designs may provide valuable insights into adaptive potential and genotype-by-environment (G × E) interactions. Previous studies have demonstrated substantial genotypic variability in *C. canephora* responses to drought, temperature, and altitude (Martins et al., 2019;

Kiwuka et al., 2023; Ferrão et al., 2024), suggesting that both phenotypic plasticity and local adaptation may shape performance across heterogeneous environments.

In this study, we investigated the establishment and early growth performance of three genetically distinct *Coffea canephora* varieties in a reciprocal transplant trial along an altitudinal gradient in the Congo Basin. Specifically, we aimed to:

1) evaluate the impact of altitudinal gradient and site-specific soil conditions on seedling growth dynamics and leaf functional trait plasticity;
2) quantify the relative contributions of genetic origin and environmental factors to trait variation, testing for significant genotype-by-environment (G × E) interactions;
3) analyze correlations and scaling relationships between growth, leaf functional, and stomatal traits; and
4) determine characterize the emergence of coordinated trait syndromes and determine their alignment with adaptive strategies along the environmental gradient.

We hypothesized that:

(1) growth and leaf functional traits would vary significantly with site conditions, while stomatal traits would show comparatively lower variation;
(2) genotypes origins would exhibit differential responses across sites, resulting in significant G × E interactions;
(3) variation in leaf morphological traits would be associated with differences in growth and biomass traits; and
(4) integrated trait syndromes would align with the environmental gradient, reflecting coordinated adaptive responses to varying site conditions.

6.4. Materials and methods

6.4.1. Sites and experimental design

The experimental sites were located at Yangambi (450 m a.s.l.; Tshopo province), Mambasa (800 m a.s.l.; Ituri province), and Mulungu (1700 m a.s.l.; South-Kivu province) in the Congo Basin (Fig. 6-1), spanning a marked altitudinal gradient. Soil properties of the experimental sites are presented in Table 6-1, while environmental characteristics are provided in Supplementary Table S6-1 and Supplementary Fig. S6-2. Yangambi and Mambasa are situated in former principal Robusta cultivation areas while Mulungu is situated in the Arabica cultivation area. Field trials were established to assess seedling establishment and growth performance across these elevations.

Three *C. canephora* varieties were included in each site: two cultivated varieties (Lula and Kwilu) and one recently introduced wild variety (Bolondi), as described in Bollen et al. (2025). The Lula variety that originate from the Lula breeding station of the DRC, is genetically related to the 'Robusta s.s.' subgroup, the Kwilu variety that originated from the Luki region of western DRC, is related to the 'Conilon/ Kouilou' subgroup, while the Bolondi is originating from a wild population in the Man and Biosphere Reserve in Yangambi in the Tshopo Province, DRC (Verleysen et al., 2023). Seeds were collected in December 2022 from the INERA (*Institut National pour l'Étude et la Recherche Agronomiques*) coffee collection in Yangambi. For each variety, ripe cherries were harvested from ten selected mother plants bearing a high number of ripe cherries. Cherries were soaked in water to remove floating fruits, manually pulped, washed, and subjected to densimetric sorting to eliminate defective seeds. After shade drying for three days, a final manual sorting was conducted to discard damaged or poorly formed seeds. A batch of viable seeds from each variety was then dispatched to each of the three experimental sites: Yangambi (450 m a.s.l.), Mambasa (800 m a.s.l.), and Mulungu (1700 m a.s.l.) (Fig. 6-1).

The trial followed a reciprocal transplant design. Seeds were directly sown on 2 February 2023 in polyethylene bags (15 cm diameter × 22 cm height) filled with locally sourced potting soil at each site. Soil was sieved before use, and no sterilization or chemical fertilization was applied. Crop management practices were kept consistent across sites. In July 2023, five months after sowing, seedlings that had reached a homogeneous height within each variety and site were selected for subsequent measurements.

At each site, approximately 40 seedlings per variety were retained for monitoring, resulting in a total sample size of 360 individuals.

Although soil was sourced locally at each site to reflect realistic agroecological conditions, we acknowledge that differences among sites therefore integrate both altitudinal climatic variation and local pedological characteristics.

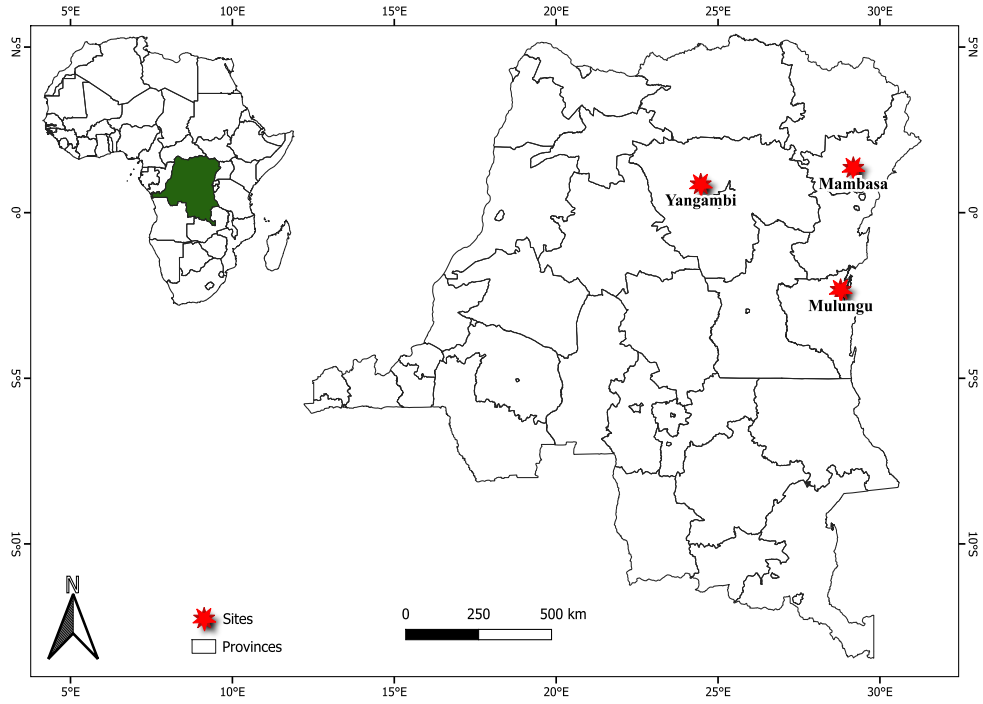

Figure 6-1. Location of the experimental sites in the East of the DR Congo.

6.4.2. Climate data of the experimental sites

Climatic data for the experimental sites were obtained using the geographic coordinates of each location from the Copernicus ERA5-Land reanalysis dataset (Muñoz-Sabater et al., 2021). ERA5-Land reanalysis data were used to provide spatially consistent and temporally continuous climatic information across all study sites, particularly in regions where long-term ground-based meteorological records are sparse, incomplete, or unavailable. Data were downloaded on 13 October 2025 from the Copernicus Climate Data Store (https://cds.climate.copernicus.eu/). Hourly surface air temperature at 2 m and accumulated total precipitation were extracted for the period January 2023 to December 2024, corresponding to the experimental timeframe. Data processing was conducted in R software version 4.5.1 (R Core Team, 2025), using the packages ncdf4 version 1.24 (Pierce, 2025), raster version 3.6-32 (Hijmans, 2025), dplyr version 1.1.4 (Wickham et al., 2023), and lubridate version 1.9.4 (Garrett and Hadley, 2011). For each site, climatic values were extracted at the exact geographic coordinates using bilinear interpolation of the raster grid. Temperature values were converted from Kelvin to Celsius, and precipitation values

were converted from meters to millimeters. Hourly temperature records were aggregated to daily means, and precipitation was summed to obtain monthly totals. Time series were visualized using ggplot2 version 3.5.2 (Wickham, 2016) and are presented in Supplementary Figure S6-2.

To contextualize recent climatic conditions, historical climatological data were obtained from WorldClim version 2.1 (1970–2000 averages; 1 km^2 resolution). Bioclimatic variables for the three experimental sites were accessed using DIVA-GIS version 7.5.0 (Hijmans et al., 2001), providing monthly mean temperature and total precipitation for the period 1970–2000 at 1 km^2 resolution. These historical data are provided in the Supplementary Table S6-1, to allow comparison between long-term climatic averages and the recent ERA5-Land conditions during the trial.

6.4.3. Soil characteristics at experimental sites

During the preparation of the seedbed, two homogenized soil samples were taken for laboratory analysis. Soil parameters were measured at the pedological laboratory of the University of Kisangani (DR Congo). Soil texture (sand, silt, clay) was determined using the pipette method (Gee and Bauder, 1986). Briefly, 50 g of air-dried and sieved soil were dispersed in 5% sodium hexametaphosphate, mechanically shaken for 16 h, and particle-size fractions were separated according to Stokes' law. The sand fraction was obtained by sieving through a 53 µm mesh, and all fractions were oven-dried and weighed. Soil pH was measured in a 1:2.5 soil-to-water suspension with a calibrated pH meter (Thomas, 1996). Total nitrogen (N) was quantified using the Kjeldahl method (Bremner, 1996), with ammonium converted to ammonia, distilled into boric acid, and quantified by titration. Nitrogen concentration was calculated as:

N (%) = (V_acid × N × 1.4) / Sample weight (g),

where V_acid is the volume of acid used for titration (mL), N is the normality of the acid, and 1.4 is the conversion factor.

Soil organic carbon (SOC) and soil organic matter (SOM) were determined using the Walkley-Black method (Walkley and Black, 1934). One gram of soil was oxidized using $K_2Cr_2O_7$ and H_2SO_4, and the remaining dichromate was titrated with $FeSO_4$. Results were corrected for incomplete oxidation using a factor of 1.32, and SOM was estimated as SOC × 1.724 (Nelson and Sommers, 1982). Cation exchange capacity (CEC) was measured using ammonium acetate saturation (Chapman, 1965), and exchangeable cations (K^+, Ca^{2+}, Na^+, Mg^{2+}) were quantified by flame photometry, with CEC calculated as $(C_{NH_4^+} \times V_{KCl})/m_{soil}$. Available phosphorus (P) was determined using the Bray II method (Bray and Kurtz, 1945). Exchangeable acidity

($S(Al^{3+} + H^{+})$) was measured following KCl extraction and titration (Thomas, 1982). Saturation of exchangeable bases (SEB) calculated as the ratio of exchangeable base cations to CEC, expressed as a percentage.

$SEB\ (\%) = [(Ca^{2+} + Mg^{2+} + K^{+} + Na^{+})/CEC] \times 100$

6.4.4. Measurement of growth traits

Seedlings' growth and functional traits were measured from July 2023 to January 2024. In July 2023, seedling height was measured using a meter ruler from the base to the apex of the plant to establish a baseline value. At the same time, initial maximum stem diameter was measured using digital calipers. In February 2024, one year after trial establishment, final plant height (FPH) and final stem diameter (FSD) were measured using the same procedures. To estimate aboveground biomass (AGB), we developed an allometric equation calibrated using *C. canephora* seedlings grown at Meise Botanic Garden, Belgium. Height and stem diameter were measured for 67 seedlings grown for 1 year and 4 months under greenhouse conditions (approximatively25°C). Seedlings were then harvested without roots, oven-dried at 65 °C for three days and weighed to determine dry aboveground biomass.

An iterative regression approach was used to identify the best-fitting relationship between plant height (H), stem diameter (D), and dry aboveground biomass. The selected model was a power function based on height and diameter:

$AGB_{(g)} = 0.0862 \cdot H^{\wedge 0.5017}{}_{(cm)} \cdot D^{\wedge 1.7536}{}_{(mm)}$; $R^2 = 0.85$ (Supplementary Figure S6-1).

Although the calibration was conducted under controlled greenhouse conditions, the model relies on structural dimensions (height and diameter) that are robust predictors of biomass in young woody plants and are expected to remain applicable across environments.

At the end of the trial, the leaf number and nodes per seedling were counted. The mean internode length of seedlings was calculated as the ratio between the final plant height and the number of nodes (Dubberstein et al., 2021; Bollen et al., 2025b). Leaf length (LL) and leaf width (LW) were measured on the largest fully expanded leaf using a graduated ruler. Leaf area (LA) was estimated using the linear model of Schmildt et al. (2015), as follows:

$LA = 0.6723 + 0.6779 * (LL * LW)$ [2]

Finally, one leaf per seedling was oven-dried for 72 hours at 65°C and weighed to determine leaf dry mass (LDM). Specific leaf area (SLA) was calculated as the ratio between leaf area and dry mass, using discs of known surface area (4.9 cm^2) obtained

with a perforator. Relative growth rate of leaf area (RGRA) was calculated following Kiwuka et al. (2023):

RGRA = ln (LA_f - LA_i)/ t_f - t_i [3]; where LA_f and LA_i represent final and initial leaf areas, respectively, and (tf - ti) corresponds to the seven-month interval between the initial and final measurements.

6.4.5. Measurement of stomatal traits

Microscopic impressions were made on the abaxial surface of dried leaves to measure stomatal traits, following the collodion method (Stojnić et al., 2015). A thin layer of colorless nail varnish was applied on both sides of the main vein. After 12 – 16 h, the dried varnish film was removed using adhesive tape and mounted on microscope slides, following Meeus et al. (2020). Photomicrographs (1,600 × 1,200 pixels; field of view = 0.09 mm^2; dimensions = 344 × 258 µm) were captured for each leaf imprint using a digital microscope (VH-5000 Ver. 1.5.1.1, Keyence Corporation, Osaka, Japan) under full coaxial illumination, with factory default shutter speed settings and an objective magnification of ×1,000 (VH-Z250R). Stomatal density (SD) was determined by manually counting the number of stomata within a 40,000 µm² grid using ImageJ v.1.54g (National Institutes of Health, USA; https://imagej.net/ij/), following Hatangi et al. (2023). Stomatal length (L) and width (W) of each stoma were measured, and stomatal surface area was calculated as π*(L/2*W/2), assuming an ellipsoidal shape (Bonal et al., 2011; Ramalho et al., 2013; Rodrigues et al., 2016). Stomatal pore size (SPS) was calculated using the same formula based on pore length and width measurements. Maximum diffusive stomatal conductance to water vapor (g_{wmax}, mol m^{-2} s^{-1}) was calculated according to Franks & Beerling (2009), as follows:

$$g_{wmax} = (\frac{d}{v}.D.a_{max})/(W + \frac{\pi}{2}\sqrt{a_{max}/\pi})$$

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

$$g_{cmax} = g_{wmax}/1.6$$

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

6.4.6. Data analysis

Statistical analyses were performed using R software version 4.5.1 (R Core Team, 2025). Prior to parametric analyses, normality of residuals was assessed using the Kolmogorov–Smirnov test, and homogeneity of variances was evaluated using Bartlett's test. When assumptions were not met, data were log-transformed. To examine the effects of site (altitude) and genetic origin on seedling traits, linear mixed-effects models (LMMs) were fitted using the *lmer* function from the lme4 package (v1.1-35.5; Bates et al., 2015). Site and origin were included as fixed effects, and their interaction (site × origin) was incorporated to test genotype-by-environment (G × E) effects, while genetic line was included as random effect. Model significance was evaluated using the *anova* function from the lmerTest package (v3.1.3; Kuznetsova et al., 2017). Marginal and conditional R^2 values (R^2_m and R^2_c) were calculated to quantify the variance explained by fixed effects and by the full model, respectively.

To further explore differences among sites, estimated marginal means (EMMs) were calculated using the emmeans package (v1.11.1; Lenth, 2025), with post hoc pairwise comparisons adjusted using Tukey's correction. Compact letter displays (CLDs) were generated using the multcomp package (v1.4.28; Hothorn et al., 2008). Trait variability across sites and origins was quantified using the coefficient of variation (CV), calculated as (standard deviation / mean) × 100.

Differences in trait variability among site-origin combinations were assessed using a permutation test for homogeneity of multivariate dispersions implemented with the betadisper function in the vegan package (v2.6.10; Oksanen et al., 2025). Analyses were conducted separately for each trait. Pairwise comparisons among groups were performed using Tukey's post hoc test (TukeyHSD), with significance letters generated via *multcompLetters* from the multcompView package (v0.1.10; Graves et al., 2024).

To explore multivariate trait structure, principal component analyses (PCA) were conducted using the FactoMineR package (v2.11; Lê et al., 2008). Spearman's rank correlations among traits were calculated and visualized using the ggcorrplot package (v0.1.4.1; Kassambara, 2016). Figures were generated using ggplot2 (v3.5.2; Wickham, 2016) and assembled using patchwork (v1.3.0; Pedersen, 2024).

6.5. Results

6.5.1. Soil properties differ among experimental sites

Soil properties differed markedly among the three experimental sites (Table 6-1). Yangambi soils were strongly acidic (pH 3.7) and characterized by high exchangeable acidity, with elevated concentrations of Al^{3+} and H^{+}, together with a relatively high cation exchange capacity (CEC) and moderate clay content. In contrast, soils at Mambasa showed moderately acidic conditions (pH 5.8), lower exchangeable acidity, and higher organic matter and soil organic carbon contents, accompanied by the highest water content among sites. Mulungu soils were near neutral (pH 7.0) and exhibited the highest clay proportion, lowest CEC, and lowest exchangeable acidity, alongside reduced organic matter and total nitrogen contents. Texturally, Yangambi and Mambasa soils were dominated by sandy and sandy-loam fractions, whereas Mulungu soils were predominantly clayey. Base saturation increased along the gradient from Yangambi to Mulungu, reflecting contrasting nutrient retention environments across sites. Bulk density decreased from Yangambi to Mulungu, corresponding to an increase in total porosity.

Table 6-1. Mean and standard deviation of soil chemical and textural properties at the three *C. canephora* experimental sites in the DR Congo

Variable	Unit	Yangambi	Mambasa	Mulungu
pH	–	3.71 ± 0.09	5.76 ± 0.05	7.04
Conductivity	µS	576.5 ± 65.76	115 ± 14.14	697.5 ± 21.92
Sand	%	50.5 ± 3.54	57 ± 1.41	12 ± 2.83
Silt	%	3 ± 1.41	11.5 ± 10.61	12.5 ± 6.36
Clay	%	46.5 ± 2.12	31.5 ± 12.02	75.5 ± 9.19
Al^{3+}	meq 100 g^{-1}	2.46 ± 0.2	1.6 ± 0.23	1.16 ± 0.85
H^{+}	meq 100 g^{-1}	2.21 ± 0.61	1.37 ± 0.42	0.61 ± 0.52
N	%	0.1 ± 0.03	0.05 ± 0.02	0.03 ± 0.02
P	ppm	76.36 ± 23.82	107.25 ± 24.62	96.02 ± 5.56
K^{+}	meq 100 g^{-1}	0.45 ± 0.07	0.34 ± 0.08	0.56 ± 0
Ca^{2+}	meq 100 g^{-1}	0.15 ± 0.04	0.18 ± 0.04	0.21 ± 0.02
Mg^{+}	meq 100 g^{-1}	0.2 ± 0.12	0.22 ± 0.06	0.4 ± 0.09
S($Al3^{+}H$)	meq 100 g^{-1}	4.68 ± 0.87	2.96 ± 0.66	1.77 ± 1.37
Soil Organic Carbon (SOC)	%	0.98 ± 0.14	2.92 ± 0.12	0.48 ± 0.18
Organic matter (OM)	%	1.69 ± 0.24	5.04 ± 0.2	0.83 ± 0.3
C/N	–	10.11 ± 3.81	65.51 ± 28.86	15.76 ± 1.86
Cation-exchange capacity (CEC)	meq 100 g^{-1}	5.47 ± 0.66	3.69 ± 0.71	2.94 ± 1.48
Saturation of exchangeable bases (SEB)	meq 100 g^{-1}	0.79 ± 0.15	0.73 ± 0.06	1.17 ± 0.11
Water content (WC)	%	8.02 ± 0.13	11.66 ± 0.69	8.21 ± 0.68
Apparent density (AD)	g cm^{-3}	1.86 ± 0.04	1.49 ± 0.05	0.99 ± 0.05
Porosity	%	30 ± 1.33	43.59 ± 1.87	62.45 ± 1.87

6.5.2. Growth traits differ among sites and origins

Linear mixed-effects models revealed significant differences in seedling development across sites depending on origin (Table 6-2, Fig. 6-2). Final plant height varied strongly among sites, decreasing with increasing altitude, whereas origin alone had no significant effect. However, the site × origin interaction was highly significant (Fig. 6-2A). Similarly, final stem diameter was strongly influenced by site, increasing with altitude, but not by origin. The interaction between site and origin was significant, indicating genotype-specific responses across sites (Fig. 6-2B). Leaf number was also significantly affected by site but not by origin, with a significant site × origin interaction (Fig. 6-2C). The internode length varied significantly among sites, decreasing with altitude increasing, whereas origin had no significant main effect, and the interaction was significant (Fig. 6-2D). Relative growth rate of leaf area (RGRA) differed significantly among sites, increasing with altitude across all origins. Origin alone had no significant effect, but the site × origin interaction was weakly significant (Fig. 6-2E). Finally, aboveground biomass (AGB) was strongly affected by site, increasing with altitude, with no significant effect of origin. The site × origin interaction was significant, indicating differential biomass accumulation across genotypes depending on site conditions (Fig. 6-2F).

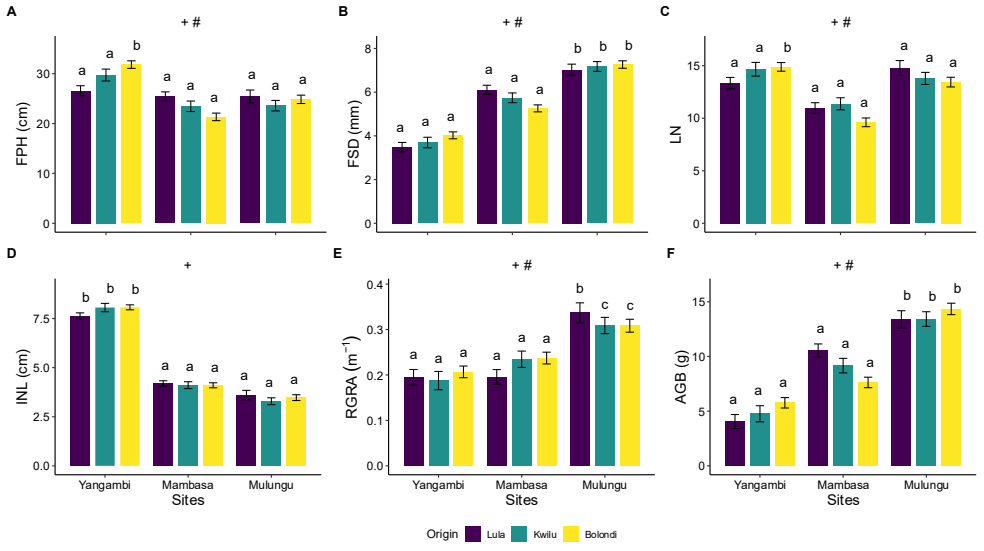

Figure 6-2. Growth and morphological traits of *Coffea canephora* seedlings by genetic origin across experimental sites at different altitudes in the eastern Democratic Republic of Congo. Bars represent means with standard errors (SE). Different letters

above bars indicate statistically significant differences ($p < 0.05$) between groups, based on pairwise comparisons using a linear mixed-effects model followed by Tukey's post hoc test (EMMEANS package). Symbols indicate significant effects of site (+), genotype origin (*) and/or their interaction (#) on the trait ($p < 0.05$). Trait abbreviations: FPH (cm), final plant height; FSD (mm), final stem diameter; LN, leaf number; INL (cm), internode length; RGRA (month^{-1}), relative growth rate in leaf area; AGB (g), aboveground biomass.

Table 6-2. F-value of results of the linear mixed-effects models analyzing the influence of site and origin of *C. canephora* seedlings on various growth traits in the Eastern DR Congo. The marginal R^2 values represent the proportion of variance explained by each fixed effect (Site, Origin and their interaction) in the LMMs. R^2_m is the variance explained by the fixed effects of the mixed effect model, while R^2_c are the total variance explained by the fixed and random effects combined in the model. Asterisks indicate the significant effects: ***is significant with $p < 0.001$, ** is significant with $p < 0.01$, * is significant with $p < 0.05$, and ns is not significant. Abbreviations: FPH (cm), final plant height; FSD (mm), final stem diameter; LN, leaf number; INL (cm), internode length; RGRA (month^{-1}), relative growth rate in leaf area; AGB (g), aboveground biomass.

Fixed effects	FPH	FSD	LN	INL	RGRA	AGB
Site	69.2***	590.78***	98.19***	789.22***	79.59***	325.94***
Origin	0.07ns	0ns	0.59ns	0.14ns	0.12ns	0.03ns
Site × Origin	19.77***	16.29***	7.59***	2.11*	2.97*	16***
R^2_m	0.39	0.77	0.4	0.82	0.3	0.66
R^2_c	0.43	0.8	0.44	0.83	0.36	0.69

6.5.3. Leaf functional and stomatal traits differ among sites and origins

Leaf functional traits were significantly influenced by both coffee genetic origin and site, with strong site × origin interactions for several traits (Table 6-3, Fig. 6-3). Leaf area (LA) varied significantly among sites, increasing with altitude, whereas origin alone had no significant main effect. However, the site × origin interaction was significant (Fig. 6-3A). Specific leaf area (SLA) differed significantly among sites, decreasing with increasing altitude, with no significant main effect of origin. The site

× origin interaction was significant, indicating genotype-specific responses to site conditions (Fig. 6-3B). Similarly, stomatal size (S), and stomatal pore size (SPS) were significantly affected by site and origin, with strong interactions between factors (Fig. 6-3C, D). Stomatal density (SD) was strongly affected by both origin and site, with a highly significant interaction, increasing with altitude (Fig. 6-3E). Finally, maximum diffusive stomatal conductance to CO_2 (g_{cmax}) did not show significant main effects of site or origin, although the interaction term was significant (Fig. 6-3F).

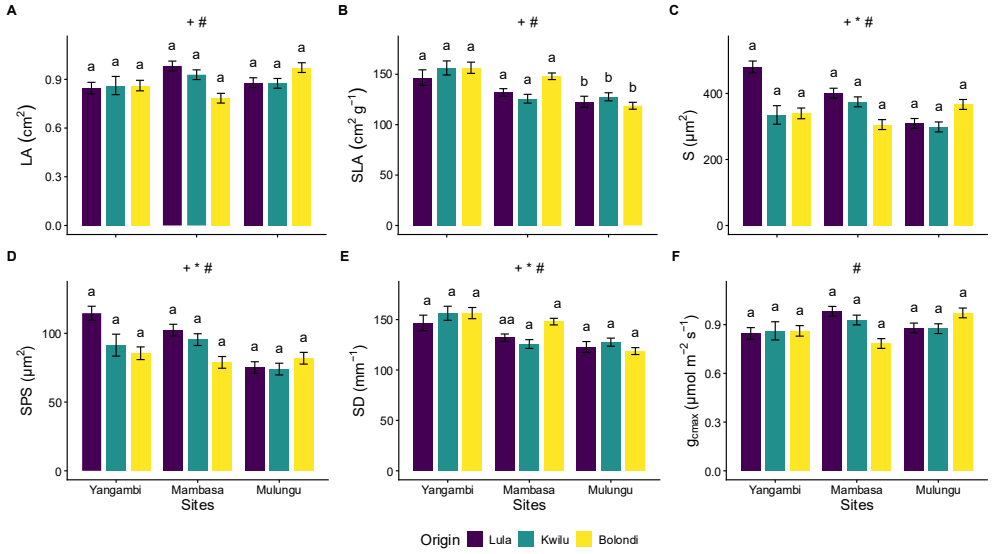

Figure 6-3. Leaf functional traits of *Coffea canephora* seedlings by genetic origin across experimental sites at different altitudes in the eastern Democratic Republic of Congo. Bars represent means with standard errors (SE). Different letters above bars indicate statistically significant differences ($p < 0.05$) between groups, based on pairwise comparisons using a linear mixed-effects model followed by Tukey's post hoc test (EMMEANS package). Symbols indicate significant effects of site (+), genotype origin (*) and/or their interaction (#) on the trait ($p < 0.05$). Trait abbreviations: LA (cm^2), leaf area; SLA ($cm^2\ g^{-1}$), specific leaf area; S (μm^2), stomatal size; SPS (μm^2), stomatal pore size; SD (mm^{-1}), stomatal density; g_{cmax} ($\mu mol\ m^{-2}\ s^{-1}$), maximum diffusive stomatal conductance to CO_2.

Table 6-3. F-value from linear mixed-effects models examining the effects of site and origin on leaf functional and stomatal traits of *C. canephora* seedlings. Site and origin were included as fixed effects, and their interaction (site × origin) was tested to assess genotype-by-environment effects. R^2_m represents the variance explained by fixed

effects, whereas R^2_c represents the total variance explained by both fixed and random effects. Asterisks indicate significance levels: *** p < 0.001, ** p < 0.01, * p < 0.05, and ns = not significant. Abbreviations: LA (cm^2), leaf area; SLA ($cm^2\ g^{-1}$), specific leaf area; S (μm^2), stomatal size; SPS (μm^2), stomatal pore size; SD (mm^{-1}), stomatal density; g_{cmax} ($\mu mol\ m^{-2}\ s^{-1}$), maximum diffusive stomatal conductance to CO_2.

Fixed effects	LA	SLA	S	SPS	SD	g_{cmax}
Site	10.14***	25.51***	11.58***	16.31***	103***	1.78ns
Origin	0.55ns	1.3ns	10.28***	7.47***	8.54***	1.01ns
Site × Origin	6.21***	6.44***	13.3***	5.41***	15.61***	7.11***
R^2_m	0.11	0.29	0.2	0.2	0.5	0.1
R^2_c	0.22	0.31	0.26	0.2	0.51	0.11

6.5.4. Site-origin effects on the dispersion of seedling morphological traits

Dispersion analysis revealed variability in seedling morphological traits differed among site-origin combinations, although the strength of these differences varied depending on the trait considered (Fig. 6). For final plant height, the dispersion analysis revealed a significant difference among groups (betadisper test: F = 2.89, p = 0.004). Pairwise comparisons indicated significant contrasts between Yangambi-Kwilu and Mulungu-Kwilu seedlings (p = 0.04) and between Mambasa-Lula and Yangambi-Kwilu seedlings (p = 0.004), while all other comparisons were not significant after Tukey correction. (Fig. 6-4A). A stronger effect was observed for final stem diameter, for which dispersion differed markedly among groups (F = 5.72, p < 0.001). Several pairwise comparisons were significant, particularly involving seedlings of Bolondi origin at Mambasa, which differed from multiple groups including Mulungu-Lula (p < 0.001), Mulungu-Kwilu (p = 0.02), and Yangambi-Kwilu (p = 0.03) (Fig. 6-4B). In contrast, variability in leaf number showed only a marginal overall difference among groups (F = 1.96, p = 0.05). However, none of the pairwise comparisons remained significant after Tukey adjustment (all p > 0.05), suggesting that differences in dispersion for this trait were comparatively weak (Fig. 6-4C).

Internode length also varied significantly across site–origin combinations ($F = 10.01$, $p < 0.001$), with pairwise contrasts particularly pronounced for seedlings from Yangambi-Bolondi and Yangambi-Kwilu compared with several other groups (e.g., Yangambi-Bolondi vs. Mulungu-Bolondi, $p < 0.001$; Yangambi-Kwilu vs. Mambasa-Bolondi, $p = 0.001$) (Fig. 6-4D). In addition, Relative growth rate (RGRA) showed a similarly strong pattern ($F = 8.28$, $p < 0.001$), with Yangambi seedlings exhibiting higher variability than most other groups, especially compared with Mambasa-Bolondi ($p < 0.001$) (Fig. 6-4E). Above-ground biomass (AGB) also differed significantly in dispersion among groups ($F = 7.61$, $p < 0.001$), with Yangambi seedlings generally showing greater variability compared with seedlings from Mulungu and Mambasa (e.g., Yangambi-Bolondi vs. Mulungu-Bolondi, $p < 0.001$; Yangambi-Kwilu vs. Mulungu-Bolondi, $p < 0.001$) (Fig. 6-4F).

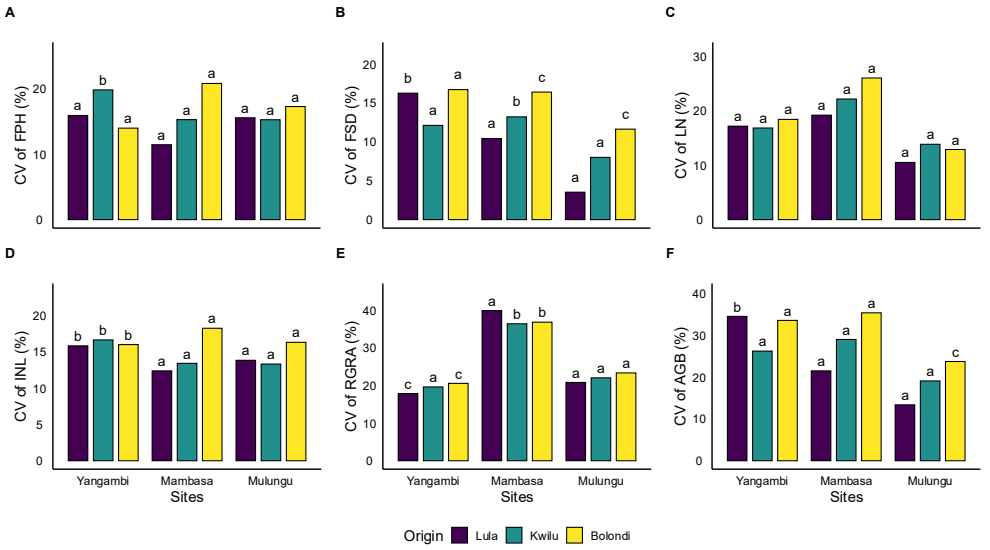

Figure 6-4. Variation in growth and morphological traits of *Coffea canephora* seedlings across combinations of genetic origin (Bolondi, Lula, Kwilu) and growth site (Yangambi, Mambasa, Mulungu). The analyzed growth and functional traits included final plant height (FPH), final stem diameter (FSD), leaf number (LN), internode length (INL), relative growth rate in leaf area (RGRA), and aboveground biomass (AGB). Dispersion among site-origin groups was assessed using betadisper, with significant differences indicated by different letters ($p < 0.05$) based on Tukey's post hoc comparisons. Thick bars represent the coefficient of variation (CV) within each group.

6.5.5. Site-origin effects on the dispersion of seedling leaf functional traits

Dispersion analysis revealed that leaf functional traits differed among site-origin combinations (Fig. 6-5). Leaf area (LA) showed a significant difference in dispersion among groups ($F = 9.08$, $p < 0.001$), with pronounced contrasts involving Mambasa-Kwilu, Mambasa-Lula, Yangambi-Lula, and Mulungu-Kwilu seedlings compared to Mambasa-Bolondi (e.g., Mambasa-Kwilu vs. Mambasa-Bolondi, $p < 0.001$; Mambasa-Lula vs. Mambasa-Bolondi, $p = 0.003$; Yangambi-Lula vs. Mambasa-Bolondi, $p = 0.014$; Mulungu-Kwilu vs. Mambasa-Bolondi, $p = 0.012$) (Fig. 6-5A). Similarly, SLA variability was significantly ($F = 3.70$, $p < 0.001$), with Yangambi-Bolondi seedlings displaying greater variation than multiple other groups, including Mulungu-Bolondi ($p = 0.005$) and Yangambi-Lula ($p = 0.014$) (Fig. 6-5B). Overall, stomatal size, and stomatal pore size were relatively conserved, as no significant differences were detected among groups (S: $F = 0.85$, $p = 0.56$; SPS: $F = 1.1$, $p = 0.36$), and post-hoc analyses did not reveal any significant contrasts between site-origin combinations (Fig. 6-5C & D). In contrast, stomatal density (SD) varied significantly among groups ($F = 3.28$, $p = 0.001$), with seedlings from Mulungu exhibiting higher densities compared to Lula seedlings grown at Yangambi, while several intermediate groups did not differ significantly (Fig. 6-5E). Similarly, maximum diffusive stomatal conductance to CO_2 (g_{cmax}) was relatively conserved ($F = 0.67$, $p = 0.71$), with no significant pairwise differences among groups (Fig. 6-5F).

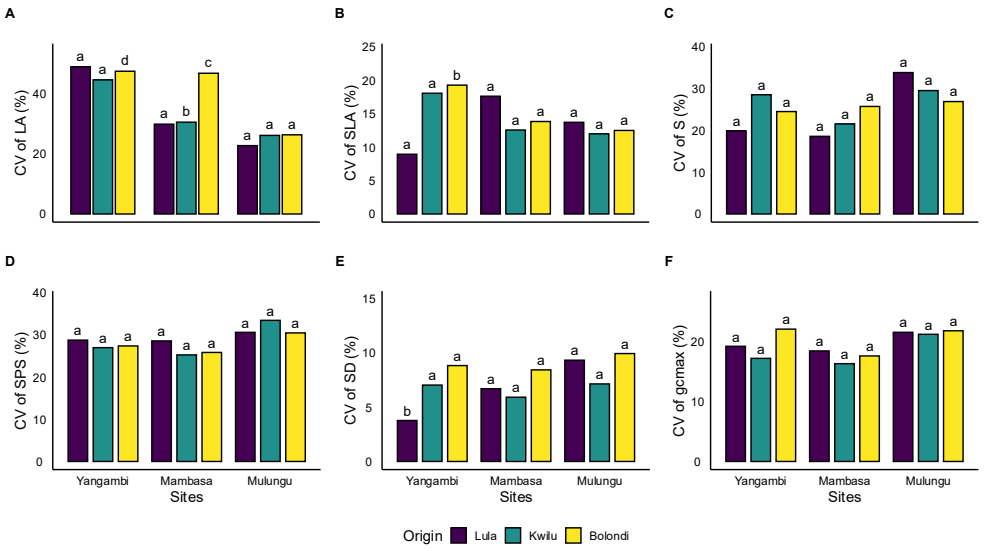

Figure 6-5. Variation in leaf functional traits of *Coffea canephora* seedlings across combinations of genetic origin (Bolondi, Lula, Kwilu) and growth site (Yangambi, Mambasa, Mulungu). The analyzed stomatal traits included leaf area (LA), specific leaf area (SLA), stomatal size (S), stomatal pore size (SPS), stomatal density (SD), and the maximum diffusive stomatal conductance to CO_2 (g_{cmax}). Dispersion among site-origin groups was assessed using betadisper, with significant differences indicated by different letters ($p < 0.05$) based on Tukey's post hoc comparisons. Thick bars represent the coefficient of variation (CV) within each group.

6.5.6. Altitudinal differentiation of integrated trait syndromes in *Coffea canephora* seedlings

The PCA biplot (Figure 6-6) revealed clear differentiation of seedling trait syndromes among sites. The first two principal components explained 60.61% of the total variance, with PC1 accounting for 39.37% and PC2 for 25.56%. PC1 was primarily associated with traits related to resource conservative strategies, including final stem diameter, internode length, aboveground biomass, stomatal pore size, specific leaf area, and stomatal size, whereas PC2 captured variation in traits related to acquisitive strategies, such as final plant height, leaf number, and maximum diffusive stomatal conductance to CO_2 (g_{cmax}). Seedlings clustered by site, with largely non-overlapping confidence ellipses, indicating distinct trait syndromes corresponding to the altitudinal gradient. Leaf area and relative growth rate in leaf area were excluded from the PCA

due to strong collinearity with final stem diameter. Spearman correlations among traits are shown in Supplementary Figure S6-3.

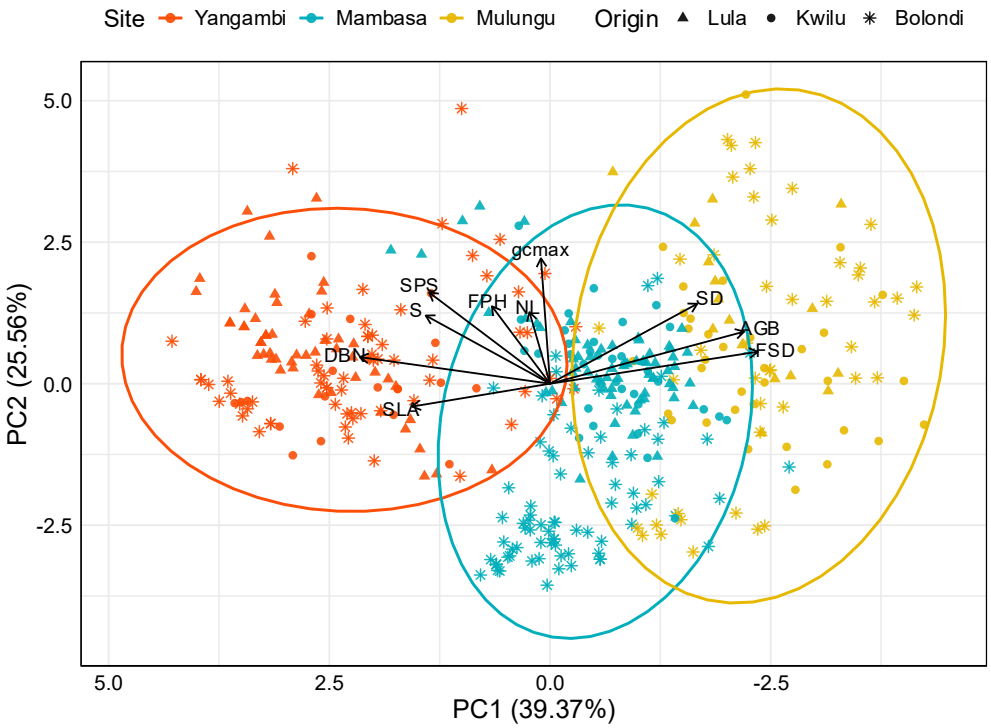

Figure 6-6. Principal Component Analysis (PCA) biplot of growth and functional traits of *Coffea canephora* seedlings across three altitudinal sites in the Eastern DRC. Individuals are colored according to site (Yangambi: red, Mambasa: blue, and Mulungu: yellow) and shaped according to origin (triangle: Lula; circle: Kwilu; asterisk: Bolondi). Ellipses represent the 95% confidence intervals for each site. Arrows indicate the direction and strength of each trait's contribution to the first two principal components (PC1 and PC2), which together explain 64.93% of the dataset's variation. PC1 primarily separates individuals based on traits related to conservative resource-use strategies including final stem diameter (FSD), internode length (INL), aboveground biomass (AGB), stomatal pore size (SPS), specific leaf area (SLA), and stomatal size (S), while PC2 is associated with traits related to acquisitive strategies, such as final plant height (FPH), leaf number (LN), and maximum diffusive stomatal conductance to CO_2 (g_{cmax}).

6.6. Discussion

Results show that traits related to vertical growth and acquisitive strategies, such as final plant height, internode length and leaf number decreased with increasing altitude, whereas traits related to resource conservative strategies, including final stem diameter, leaf area, relative growth rate of leaf area, and aboveground biomass increased with elevation, indicating shifts in growth strategy along the altitudinal gradient. Although origin effects were generally weak when considered alone, they were frequently expressed through significant site × origin interactions, highlighting genotype-specific responses to environmental conditions. Pronounced morphological differentiation among site-origin combinations was observed, particularly for seedlings grown at Yangambi, while within-site contrasts were often minimal. Stomatal traits, although responsive in several univariate analyses, displayed comparatively moderate differentiation at the multivariate level, with stomatal density emerging as the most consistently site-sensitive anatomical trait. Overall, these findings indicate that seedling performance in *C. canephora* is primarily driven by phenotypic plasticity and environmental filtering, with secondary modulation by genotype-dependent responses.

6.6.1. Site-driven variation in seedling growth and morphology reflects environmental adaptation

Our results show that altitude acts as a strong environmental filter shaping early seedling development in *C. canephora*. Seedling height declined with elevation, consistent with cooler temperatures and reduced atmospheric pressure constraining shoot elongation at higher altitudes (Barbosa et al., 2014). Nevertheless, seedlings grown at high altitude in Mulungu attained heights comparable to those at low elevation and developed larger stem diameters. Similar responses have been reported where *C. canephora* is cultivated within moderate thermal ranges, provided minimum temperatures exceed ~17 °C and maximum remain below 34 °C (Partelli et al., 2013; Barbosa et al., 2014). Mean temperatures at Mulungu (~17 °C) remained above thresholds associated with photosynthetic impairment (<13–14 °C), indicating that thermal limitation was not yet restrictive (Partelli et al., 2013). These findings suggest that altitude constrained elongation without preventing establishment, supporting evidence that *C. canephora* has broader thermal tolerance than traditionally assumed.

Although genetic origin alone did not affect plant height, the significant site × origin interaction indicates genotype-specific responsiveness to environmental conditions, consistent with phenotypic plasticity or local adaptation (Martins et al., 2019). While

C. canephora is generally considered more resilient and cost-efficient to cultivate than *C. arabica* (DaMatta and Ramalho, 2006), substantial genotypic variation in performance under cooler conditions has been documented. Several genotypes perform well at mean temperatures around 20 °C and minimum temperatures between 10 and 20 °C (Martins et al., 2019), in line with thermal optima proposed by Kath et al. (2020). These estimates contrast with earlier reports suggesting higher optima (22–30 °C; DaMatta and Ramalho, 2006) but align with experimental evidence indicating that *C. canephora* combines relative heat tolerance with sensitivity to prolonged stress (Rodrigues et al., 2016; DaMatta et al., 2018). Our results thus provide initial evidence that Congolese *C. canephora* genotypes can establish at elevations up to 1700 m, although long-term productivity remains to be assessed.

Structural and leaf functional traits related to resource-acquisitive strategies responded coherently to altitude. The increase in final stem diameter with elevation suggests a shift toward greater investment in mechanical support and carbon storage, likely reflecting adaptation to cooler temperatures and increased wind exposure, consistent with previous studies (Moraes et al., 2020; Senra et al., 2023). Contrasting trends reported elsewhere highlight the role of local edaphic and microclimatic conditions (Pangestika et al., 2025). Leaf number also increased at higher altitude, potentially compensating for reduced metabolic rates by maintaining photosynthetic surface area (Moraes et al., 2020). However, inconsistencies documented by Ahmed et al. (2021) indicate that leaf production emerges from complex interactions among climatic, edaphic, and genetic controls rather than a unidirectional altitudinal response.

Above-ground biomass increased with altitude and showed significant genotype × environment interactions, indicating that biomass accumulation is shaped by both site conditions and genotype-specific plasticity. Similar non-linear altitude-biomass relationships have been reported in tropical and montane ecosystems, where biomass often increases with elevation up to a threshold before declining under harsher conditions (Maza et al., 2022; Hu et al., 2025). In *C. canephora*, these patterns likely reflect coordinated adjustments in growth and allocation, with genotype selection playing a key role in optimizing productivity across elevation zones (Martins et al., 2019). Altitude-driven shifts in soil microbial communities may further modulate biomass accumulation via effects on nutrient availability (Tapaça et al., 2024). The observed increase in biomass with altitude can be linked to a transition from strongly acidic, Al-rich soils at lower elevations, where phosphorus availability and root development are constrained, toward more favorable edaphic conditions. Under such acidic conditions, *Coffea* depends heavily on arbuscular mycorrhizal fungi (AMF) for

nutrient acquisition, although extreme acidity can limit AMF diversity and colonization efficiency (Jansa et al., 2014; Marro et al., 2022). Our results suggest that biomass variation along the altitudinal gradient reflects a shift toward more efficient soil-plant-microbe interactions, with genotype-specific responses likely driven by differences in mycorrhizal dependency and nutrient acquisition strategies.

Specific leaf area declined sharply with altitude, reflecting the development of thicker, denser leaves under low temperatures, high radiation, and increased wind exposure (Körner et al., 1986; Barbosa et al., 2014). This response reflects altitudinal trait syndromes documented across temperate and tropical tree species, where conservative leaf traits dominate at higher elevations (Walther et al., 2002; Hoch and Körner, 2005; Körner, 2007; Read et al., 2014). Comparable responses have been observed in *C. arabica* (Worku et al., 2018; Moraes et al., 2020). Soil properties also influence trait expression: in elevational studies, soil nutrient availability, pH, and texture are associated with variation in SLA and related traits, although climatic drivers often have stronger effects (X. Zhang et al., 2024). Nutrient-poor soils with high acidity at lower elevations can constrain leaf expansion and favor thinner leaves, whereas more balanced nutrient conditions at mid-elevations support greater nutrient uptake and investment in thicker, denser leaves. Together, our results indicate that *C. canephora* seedlings express coordinated trait syndromes that facilitate establishment across a broad altitudinal range, mediated by strong genotype-by-environment interactions. This trait-based perspective provides a mechanistic basis for identifying genotypes suited to cooler and more variable environments and supports breeding and agroecological strategies aimed at enhancing coffee resilience under climate change.

6.6.2. Leaf traits and physiological implications

We observed that leaf area increased with altitude, potentially optimizing light capture in cooler, shaded environments at the Mulungu site. Our results contrast with findings by Ahmed et al. (2021), which showed some genotypes experienced no change or even a reduction in leaf area at higher elevations, possibly due to drought stress or nutrient limitations. Our results are in contradiction with many other studies, which showed that leaf size decreased with latitude for several species, although there is variation among species (Li et al., 2020). They are also in contradiction with the findings of Guo et al. (2018), who found that leaf area increased for Bamboo (*Pleioblastus amarus*) with altitude increasing in China. Several studies have shown that altitude impacts leaf morphology variably, with reductions in leaf size. Ke et al. (2022) found a significant reduction in leaf size at higher altitudes across 39 broad-leaved herbaceous species on the northeastern Qinghai-Tibetan Plateau. The decrease was mainly attributed to reduced leaf length rather than width, linking these changes

to leaf energy balance under cooler, high-altitude conditions. Soil fertility influences leaf traits by affecting nutrient availability, often resulting in leaves with higher dry matter content but lower nitrogen in nutrient-poor soils (Jin et al., 2023). Water availability strongly affects leaf size and quality, as moisture stress typically reduces leaf length and area, while genotypic differences modulate drought tolerance and the plasticity of leaf traits (Poorter et al., 2009; Ismael et al., 2022; Kiwuka et al., 2023). Genetic factors also play key roles; for instance, specific loci in maize control leaf number above and below the primary ear, while leaf growth in coffee varies according to axis order and vertical position (Brown et al., 2011; Rakocevic and Matsunaga, 2018). The relative growth rate of leaf area (RGRA) followed a similar altitude-dependent increase, facilitating rapid development. This pattern is consistent with findings that genotypic plasticity and environmental factors strongly influence leaf expansion rates (Poorter et al., 2009; Scheepens et al., 2010) and that nutrient-related leaf traits exhibit high plasticity along elevational gradients (Zhang et al., 2024). However, RGRA responses can vary according to genotype and local stress conditions, as observed in drought-sensitive genotypes of birch and *C. canephora* populations across different sites (Aspelmeier and Leuschner, 2004; Kiwuka et al., 2023), highlighting the interplay between genetic potential and environmental constraints.

The variation in stomatal traits among *C. canephora* origins and growth sites, which also showed a pronounced genotype-by-environment interaction. This result is consistent with prior research showing that stomatal characteristics are plastic and respond to environmental gradients (Hetherington and Woodward, 2003; Wang et al., 2014). Studies along elevational gradients report increased stomatal density (SD) at higher altitudes or under harsher conditions, linked to adaptations for gas exchange and thermal regulation (Carlson et al., 2016; Liu et al., 2020; Li and Prentice, 2024). In *Protea repens*, stomatal density increased in hotter, drier source populations and correlated with higher photosynthetic rates, indicating adaptive differentiation (Carlson et al., 2016). Recent meta-analysis findings confirm that stomatal density and index are more responsive to light intensity and temperature than CO_2 concentration alone (Poorter et al., 2025). Moreover, a recent study of high-altitude plant adaptation emphasizes that phenotypic variation in leaf anatomical and stomatal traits underpins survival in extreme environments (Zhang et al., 2024). In the current study, stomatal size and stomatal pore size decreased with altitude, reflecting a possible strategy to reduce water loss under harsher environmental conditions while maintaining controlled gas exchange. Although a trade-off between stomatal size and density has been widely reported in coffee and other species (Rodrigues et al., 2018;

Poorter et al., 2025), whereby smaller stomata are often associated with higher densities, this pattern was not observed here. Stomatal size and density both decreased with altitude, indicating a shift toward more conservative gas exchange (Zhang et al., 2020; Ayala-Ramos et al., 2024). This decline in density contrasts with reports of increased stomatal density at higher elevations, suggesting instead a strategy of reduced gas exchange under cooler, resource-limited conditions (Woodward and Kelly, 1995; Körner, 2007; Carlson et al., 2016). In our study, maximum diffusive stomatal conductance to CO_2 (g_{cmax}), in contrast to the clear altitudinal responses of individual anatomical traits, did not show a consistent main effect of altitude but exhibited significant genotype-by-environment interactions. This result suggests compensatory mechanisms where different stomatal traits balance each other to sustain photosynthetic function across environments. Similar patterns have been reported in coffee (Rodrigues et al., 2018), and in the *Brachypodium distachyon* complex, where inverse scaling between stomatal size and density preserves conductance despite structural divergence (Berg et al., 2025).

6.6.3. Trait states reflect altitudinal environmental constraints in *C. canephora* seedlings

Our findings show that *C. canephora* seedlings express coordinated adjustments in growth, leaf, and stomatal traits across an altitudinal gradient. The clear segregation of individuals in PCA space along axes associated with acquisitive versus conservative strategies, together with strong correlations and trade-offs among functional traits, indicates that altitude structures integrated, multivariate trait syndromes rather than eliciting independent trait responses. Seedlings from low-altitude Yangambi expressed acquisitive traits: larger stomata and higher SLA, typically favored in warm, resource-rich environments (Wright et al., 2004; Poorter et al., 2009). Larger stomatal structures and higher potential stomatal conductance enhance carbon uptake, supporting rapid growth and high productivity (Franks and Beerling, 2009). Similar patterns of acquisitive trait expression at low altitude have been documented in tropical trees (Fyllas et al., 2014) and in other *Coffea* species (DaMatta et al., 2018). At high altitude (Mulungu), seedlings exhibited a suite of traits indicative of a conservative resource-use strategy, including reduced stomatal size and density, lower SLA, and shorter internodes. These traits are commonly associated with environments characterized by lower temperatures and constrained resource acquisition, where growth and carbon assimilation rates are limited (Reich et al., 2003; Körner, 2007; Umaña and Swenson, 2019; Yang et al., 2022). Conservative investment in thicker leaves and reduced stomatal dimensions is known to enhance water-use efficiency, maintain hydraulic safety, and improve leaf longevity under low

temperatures (Niinemets, 2010). For *C. canephora*, which naturally spans a broad ecological range, these results highlight substantial phenotypic flexibility in response to altitude.

The observed negative relationships between internode length and both final stem diameter and biomass reflect a classic trade-off between vertical elongation and radial thickening (King, 1998; Sterck and Bongers, 2001; Jucker et al., 2025). Taller seedlings with longer internodes likely favor light capture, whereas thicker stems support mechanical stability and carbon storage. Such trade-offs are well established in tropical tree seedlings and appear to operate similarly in *C. canephora* (Poorter et al., 2015). The negative relation between SLA and above-ground biomass aligns with global leaf economic spectrum patterns, where low-SLA (thicker, denser) leaves support long-lived, structurally robust tissues and slower but more efficient growth (Wright et al., 2004). This coordination between leaf economics and whole-plant performance reinforces the idea that altitude reshapes not only individual traits but broader resource-acquisition strategies.

We found that stomatal size, pore size, and diffusive stomatal conductance to CO_2 (g_{cmax}) were strongly correlated, reflecting well-established biophysical constraints linking stomatal geometry to gas-exchange capacity (Franks and Farquhar, 2007; Dow et al., 2014). Low-altitude seedlings with larger stomata likely benefit from enhanced carbon assimilation under high temperature and high evaporative demand (Hetherington and Woodward, 2003). Conversely, reduced stomatal sizes at high altitude are consistent with lower transpiration demand and reduced metabolic rates (Körner and Paulsen, 2004). The positive relationship between stomatal density and biomass suggests that seedlings with greater growth potential invest in greater gas-exchange capacity, a pattern also observed in other tropical crops (Damour et al., 2010). The negative correlation between stomatal density and SLA points to coordinated adjustments toward thicker, longer-lived leaves, traits advantageous under cooler, high-altitude conditions. Altitudinal differentiation in trait syndromes suggests strong phenotypic plasticity or localized adaptation in *C. canephora* populations. As climate change shifts temperature regimes across the Congo Basin, identifying trait combinations linked to performance across environments becomes increasingly relevant. Low-altitude acquisitive phenotypes may be better suited to warmer, wetter climates, while conservative high-altitude phenotypes may be favored in cooler or water-limited environments (DaMatta and Ramalho, 2006; Davis et al., 2012). These findings provide a functional basis for guiding planting material selection, particularly in agroforestry systems expanding into higher altitudes or marginal lands. Further reciprocal-transplant or genomic studies would help

disentangle plastic vs. genetic components of these responses and strengthen understanding of the adaptive capacity of *C. canephora*.

6.6.4. G × E interaction and the role of pedological conditions

Beyond the dominant effect of altitude, our results clearly demonstrate the importance of genotype-by- environment (G × E) interactions in shaping growth, morphology, and leaf functional traits of *Coffea canephora* seedlings. Although origin alone explained only a limited proportion of trait variation, significant G × E effects were consistently detected across multiple traits, indicating that genotypes responded differently depending on local environmental conditions. Similar patterns have been widely reported in coffee and other perennial crops, where phenotypic expression is strongly context-dependent rather than genetically fixed (Martins et al., 2019; Kath et al., 2020). Pedological heterogeneity among sites likely played a key role in reinforcing these G × E interactions. Soils at Yangambi are predominantly deeply weathered Ferralsols, typically characterized by low nutrient availability but good structure and drainage, conditions known to favor rapid early growth under warm and humid climates (Quesada et al., 2010; Nottingham et al., 2015). In contrast, high-altitude soils at Mulungu are characterized by neutral pH, high clay content, and elevated base cation availability, which can enhance nutrient retention despite relatively low organic matter levels. These edaphic conditions likely promote greater stem thickening and biomass accumulation, even under cooler temperatures, by improving nutrient availability and reducing aluminum toxicity (Körner, 2007; Guo et al., 2024; Tapaça et al., 2024). Soils at Mambasa represent an intermediate pedological context, combining relatively high organic matter with moderate nutrient availability and stable moisture conditions, which may buffer environmental stress. These edaphic characteristics are likely to interact with genotype-specific nutrient acquisition strategies, thereby amplifying G × E effects (Poorter et al., 2015).

In our study, traits showing strong G × E interactions, including final stem diameter, leaf area, relative growth rate of leaf area, and aboveground biomass, were particularly sensitive to soil nutrient availability and physical constraints. Genotypes that performed well at one site did not necessarily maintain their relative ranking across sites, suggesting differential capacities for nutrient acquisition, carbon allocation, and root-shoot balance. Comparable genotype-specific responses to edaphic variability have been reported for *C. canephora* by Martins et al. (2019) and for other tropical woody species by Maza et al. (2022), highlighting the central role of soil-climate interactions in shaping productivity. By contrast, stomatal traits exhibited weaker G × E effects, consistent with the idea that anatomical traits are more constrained by

developmental and biophysical limits (Franks and Farquhar, 2007; Dow et al., 2014). Nevertheless, the significant site-dependent variation in stomatal density observed in our study suggests that soil-driven differences in water and nutrient availability may indirectly influence gas-exchange strategies, as also proposed by Hetherington and Woodward (2003) and Poorter et al. (2022). Overall, our findings reinforce that altitudinal climate gradients alone cannot fully explain variation in *C. canephora* seedling performance. Instead, genotype-by-environment interactions mediated by pedological heterogeneity play a central role in determining trait expression and growth strategies. Integrating soil information into genotype evaluation and selection frameworks is therefore essential for identifying robust planting material and improving the resilience of coffee cultivation under increasing environmental variability.

6.7. Conclusion

This provides a comprehensive assessment of the adaptive performance of three *Coffea canephora* varieties grown across sites located along a broad altitudinal gradient in the Congo Basin, revealing pronounced phenotypic differentiation associated with site conditions, genetic origin, and their interaction. Seedlings exhibited clear shifts in growth, functional, and stomatal traits along the gradient reflecting coordinated adjustments in growth strategy and physiology during early development. Traits associated with acquisitive strategies declined with increasing altitude, whereas traits linked to conservative strategies increased, indicating a shift toward more conservative growth under cooler conditions. Stomatal trait variation further suggests fine-scale physiological adjustments supporting gas exchange under contrasting environmental conditions. Performance differed among varieties across environments, highlighting genotype-specific responses and suggesting that the introduction and conservation of wild genetic diversity could substantially enhance adaptive potential. Overall, the observed patterns reveal considerable phenotypic plasticity, enabling Congolese *C. canephora* seedlings to maintain performance across contrasting altitudes. However, longer-term observations are required to evaluate productivity, yield stability, and reproductive performance. Such information will be essential for guiding breeding, conservation, and agroforestry strategies aimed at improving resilience, sustaining productivity, and ensuring the long-term cultivation of this economically important species under ongoing environmental change.

6.8. Data availability statement

Data that support the findings of this study are available on Zenodo at https://doi.org/10.5281/zenodo.19511263.

6.9. Funding information

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6.10. Acknowledgments

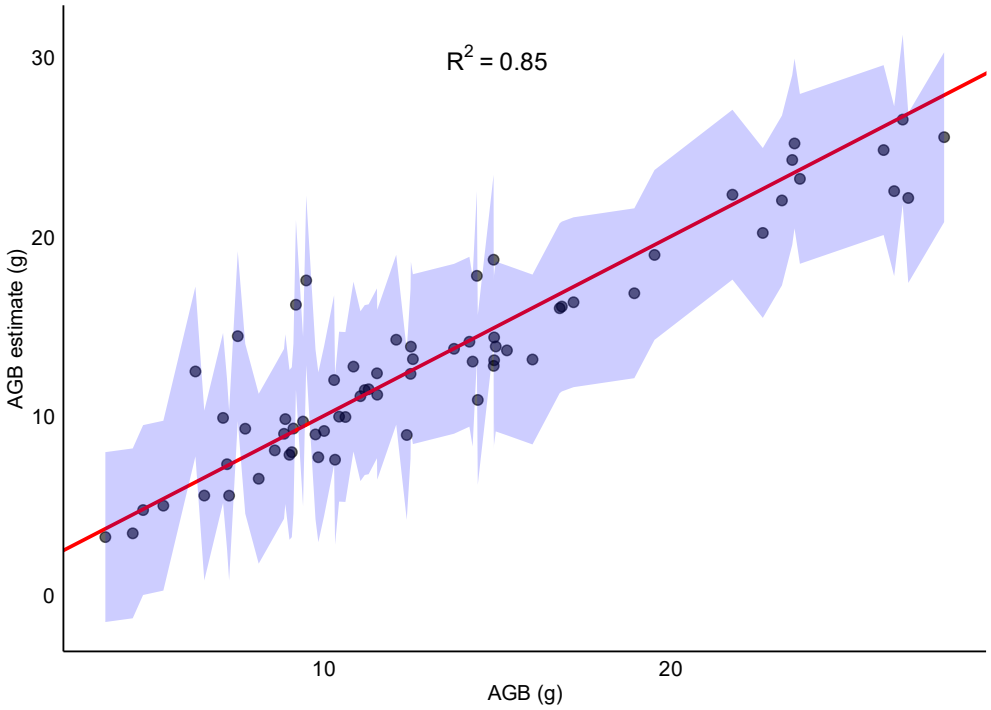
We thank the institutions that provided financial support, namely the Meise BG, RBINS, ARES, and CIFOR-ICRAF.

6.11. Conflict of interest

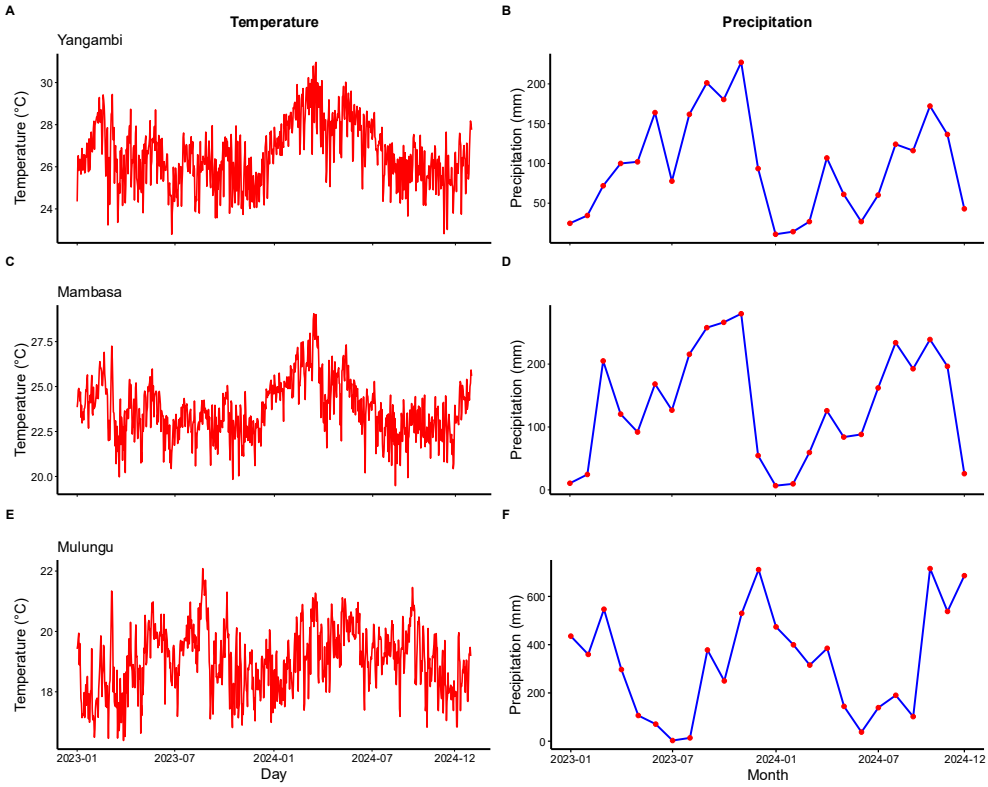
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6.12. Supplementary materials

6.12.1. Supplementary results



Supplementary Figure S6-1. Relationship between observed aboveground biomass (AGB) and model-estimated AGB for *Coffea canephora* seedlings using the power model. The red line represents the 1:1 relationship, the blue shaded area indicates the 95% confidence interval, and the coefficient of determination (R^2) is shown on the plot.



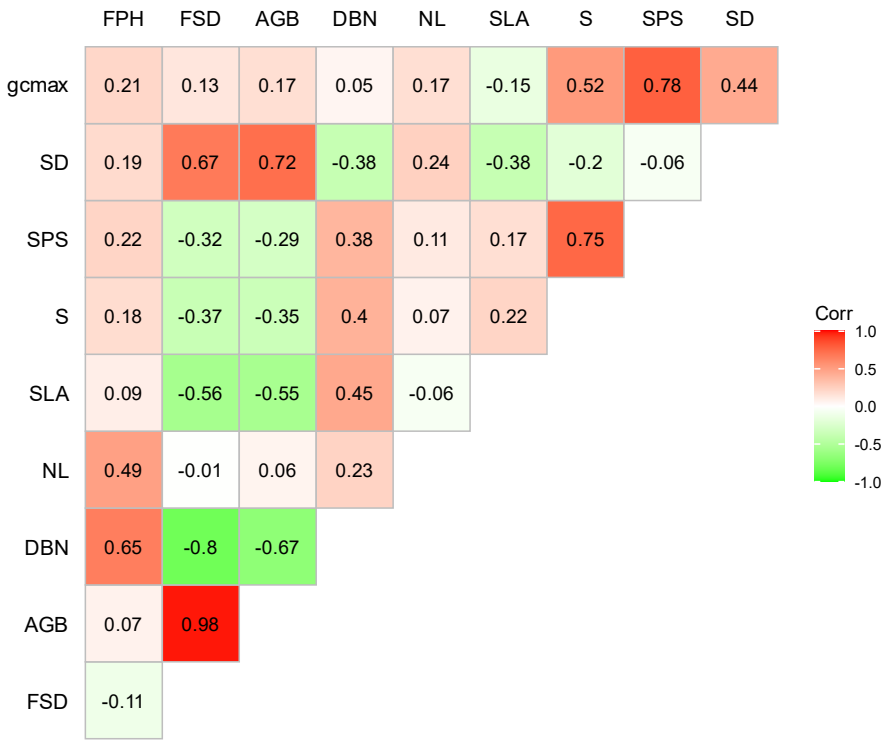
Supplementary Figure S6-2. Daily mean temperature and monthly total precipitation at the three experimental sites in the Democratic Republic of Congo.

Supplementary Table S6-1. Bioclimatic variables of the three *C. canephora* experimental sites located in the Eastern part of the Democratic Republic of Congo.

Variable	Unit	Yangambi	Mambasa	Mulungu
Mean Annual Temperature	°C	24.59	23.82	17.66
Mean Diurnal Range	°C	9.92	11.73	10.20
Isothermality	–	85.49	84.96	83.61
Temperature Seasonality	–	45.77	45.25	36.55
Max Temperature of Warmest Month	°C	30.8	31	23.6
Min Temperature of Coldest Month	°C	19.2	17.2	11.4
Temperature Annual Range	°C	11.6	13.8	12.2
Mean Temperature of Wettest Quarter	°C	24.38	23.23	17.68
Mean Temperature of Driest Quarter	°C	24.70	24.02	17.22
Mean Temperature of Warmest Quarter	°C	25.15	24.37	17.90
Mean Temperature of Coldest Quarter	°C	24.02	23.23	17.20
Annual Precipitation	mm	1776	1606	1627
Precipitation of Wettest Month	mm	229	184	191
Precipitation of Driest Month	mm	73	62	24
Precipitation Seasonality	mm	30.46	29.65	45.61
Precipitation of the Wettest Quarter	mm	612	504	551
Precipitation of the Driest Quarter	mm	279	224	120
Precipitation of Warmest Quarter	mm	417	393	353
Precipitation of Coldest Quarter	mm	478	504	196

Spearman correlation analysis revealed strong connections among growth and functional traits of *C. canephora* seedlings (Supplementary Figure S6-3). Final stem diameter (FSD) and aboveground biomass (AGB) showed a robust positive connection ($r = 0.98$, $p < 0.001$), indicating that stem thickening closely reflects biomass accumulation. Final plant height (FPH) was moderately connected with the distance between nodes (DBN) ($r = 0.65$, $p < 0.001$) and the number of leaves ($r = 0.49$, $p < 0.001$), suggesting that taller seedlings tend to develop longer internodes and produce more leaves. In contrast, DBN showed negative connections with FSD ($r = –0.80$, $p < 0.001$) and AGB ($r = –0.67$, $p < 0.001$), reflecting trade-offs between vertical elongation and radial growth.

Leaf functional traits were also strongly connected. Specific leaf area (SLA) showed negative connections with FSD and AGB ($r = –0.55$, $p < 0.001$), indicating that seedlings investing in denser, thicker leaves tend to accumulate more biomass. Stomatal traits displayed tight interconnections: stomatal size (S) and pore size (SPS) were closely linked ($r = 0.75$, $p < 0.001$), and both were positively connected to maximum stomatal conductance to CO_2 ($r = 0.52$-0.78, $p < 0.001$). Stomatal density (SD) showed an opposite pattern, being negatively connected with SLA ($r = –0.38$, $p < 0.01$) but positively with FSD ($r = 0.67$, $p < 0.001$) and AGB ($r = 0.72$, $p < 0.001$). Together, these connections highlight a coordinated network between growth performance, structural investment, and gas exchange capacity, reflecting integrated trait strategies across altitudinal gradients.



Supplementary Figure S6-3. Heatmap of Spearman's correlation coefficients among key morphological and growth traits. Only the upper triangle of the correlation matrix is shown, with correlation values displayed on the squares. Colors range from green (negative correlation) through white (no correlation) to red (positive correlation). The legend indicates the strength and direction of the correlations. Traits included are Final plant height (FPH), final stem diameter (FSD), number of leaves (NL), distance between nodes (DBN), aboveground biomass (AGB), specific leaf area (SLA), stomatal pore size (SPS), stomatal density (SD), and maximum diffusive stomatal conductance to CO_2 (g_{cmax}).

6.12.2. Supplementary Methods for soil data calculation

Since local soil mixtures were used in the trial, soil parameters were considered at the same level as climatic ones. All edaphic parameters were measured in the pedological laboratory of the Faculty of Renewable Natural Resource Management of the University of Kisangani (DR Congo) using two homogenized soil samples taken from each experimental site.

Soil texture was determined by measuring the particle size distribution of clay, silt, and sand fractions using the pipette method, following the protocol described by Gee and Bauder (1986).

A Robinson Pipette (Apparatus Pipette, SDEC, Solutions Technologiques pour l'Environnement, Tours, France) was used for the measurements. In short, approximately 50 grams of air-dried and sieved soil (<2 mm) were placed in a beaker and dispersed in a 5% solution of sodium hexametaphosphate (Calgon, $(NaPO_3)_6$) as a dispersing agent. The mixture was mechanically shaken for 16 hours to ensure full dispersion of aggregates. The suspension was then transferred to a 1-liter sedimentation cylinder and filled to the mark with distilled water. Based on Stokes' law, aliquots were drawn at predetermined depths and time intervals using a pipette to isolate silt and clay fractions. Each aliquot was transferred into a pre-weighed beaker, oven-dried at 105°C for 24 hours and weighed. The sand fraction was determined by washing the remaining suspension through a 53 µm sieve, drying, and weighing the residue. The percentages of sand, silt, and clay were calculated from the dry weights of each fraction.

Soil pH was measured by potentiometry using a calibrated pH meter (Hanna Hi 9124, Hanna Instruments, Strasbourg, France), following the procedure described by Thomas (1996). For each sample, 10 grams of air-dried, sieved soil (<2 mm) were mixed with 25 ml of deionized water in a 1:2.5 soil-to-water ratio (w/v). The suspension was stirred thoroughly and allowed to equilibrate for 30 minutes. The pH was then measured by immersing a glass electrode in the supernatant. The pH meter was calibrated using standard buffer solutions at pH 4, 7, and 10 before each set of measurements. All measurements were conducted at room temperature (approximately 25 °C, in the Kisangani region).

Total nitrogen (N) content in the soil was determined using the Kjeldahl method, following the procedure outlined by Bremner (1996). In this method, approximately 1 gram of finely ground, air-dried soil was digested with concentrated sulfuric acid (H_2SO_4) in an apparatus Kjeldahl (Omnilab Foodalyt D1000, Hanon Group, Jinan, China), in the presence of a catalyst mixture containing selenium sulfate and potassium sulfate, to accelerate the reaction. During digestion, organic nitrogen in the soil was converted to ammonium (NH_4^+). After digestion, the solution was cooled and transferred to a distillation apparatus. Sodium hydroxide (NaOH) was added to the digest to neutralize the acid and convert ammonium ions (NH_4^+) into ammonia (NH_3). Ammonia was distilled off and collected in a boric acid (H_3BO_3) solution, where it formed ammonium borate. The H_3BO_3 solution containing the ammonia was titrated

with 0.01 M hydrochloric acid (HCl) until the endpoint was reached. The volume of acid used in the titration was directly proportional to the amount of nitrogen in the sample. Total nitrogen content was calculated using the following formula:

$$Nitrogen\ (\%) = \frac{V_{acid}\ x\ N\ x\ 1.4}{Sample\ weight\ (g)} \qquad [1]$$

where:

- V_{acid} = volume of acid used in titration (ml),
- N = normality of the acid,
- 1.4 is a constant that converts titration results to nitrogen percentage in the sample.

Soil organic carbon (SOC) and Total soil organic matter (SOM) were determined using the Walkley and Black wet oxidation method (Walkley and Black, 1934). To achieve this, 1 g of air-dried, finely ground soil was treated with 10 ml of 1 N potassium dichromate ($K_2Cr_2O_7$) solution, followed by 20 ml of concentrated sulfuric acid (H_2SO_4), using an Erlenmeyer flask (Duran Group, Mainz, Germany). The acid-generated heat causes the oxidation of organic carbon in the soil. The flask was gently swirled to ensure complete mixing. The mixture was allowed to rest for 30 minutes to ensure complete oxidation. After cooling, the mixture was diluted with 200 mL of distilled water. 10 ml of orthophosphoric acid (H_3PO_4) was added to suppress the interference from iron and manganese in the soil. Then, 1 ml of diphenylamine indicator was added. The excess potassium dichromate was titrated with 0.5 N ferrous sulfate ($FeSO_4$) solution until the color changed from violet to green, indicating the endpoint. A blank was run alongside the sample, following the same procedure but without soil. This was necessary to account for the inherent oxygen demand of the reagents. The organic carbon was calculated based on the difference between the amount of dichromate used in the blank and the amount used in the sample according to this equation:

$$Soil\ Organic\ Carbon\ (\%) = \frac{(V_{blank}\ - V_{sample}\ )\ x\ N\ x\ 0.003\ x\ 100}{Sample\ weight\ (g)} \qquad [2]$$

where:

- V_{blank} = volume of ferrous sulfate used for the blank (ml),
- V_{sample} = volume of ferrous sulfate used for the sample (ml),
- N = normality of ferrous sulfate,

- 0.003 = equivalent weight of carbon in grams per mole of dichromate.

Since the Walkley and Black method does not fully oxidize all organic carbon, a correction factor of 1.32 was applied to account for incomplete oxidation and to more accurately estimate total organic carbon (Nelson and Sommers, 1982). Total soil organic matter (SOM) was then estimated by multiplying the corrected SOC values by 1.724, based on the assumption that organic matter contains 58% carbon (Nelson and Sommers, 1982).

The Cation Exchange Capacity (CEC) of soil samples was determined using the ammonium acetate saturation method, followed by quantification of exchangeable cations via flame photometry (Chapman, 1965). In this method, 10 g of air-dried, sieved (<2 mm) soil was saturated with 1 M ammonium acetate solution (CH_3COONH_4) with pH 7 by shaking the mixture for 1 hour. The samples were then centrifuged, and the supernatant was discarded. Excess ammonium acetate was removed by washing the soil with 95% ethanol. Exchangeable ammonium ions were subsequently displaced using 1 M potassium chloride (KCl), and the resulting filtrate was collected. The concentrations of exchangeable potassium (K^+), calcium (Ca^{2+}), sodium (Na^+), and magnesium (Mg^{2+}) were measured using a JENWAY PFP7 flame photometer (Comate, Engineering and Design, Leuven, Belgium). CEC was calculated by determining the concentration of displaced ammonium ions in the supernatant, using the following formula:

$$\text{CEC (cmol(+) kg}^{-1}) = \frac{C_{NH_4^+} \times VKCl}{m_{soil}} \qquad [3]$$

where $C_{NH_4^+}$ is the concentration of displaced ammonium ions (in cmol/L), VKCl is the volume of KCl solution used for displacement (in L), and m_{soil} is the mass of the soil sample (in kg). CEC was expressed in milliequivalents per 100 g (meq 100 g^{-1}), which is numerically equivalent to cmol(+) kg^{-1}.

Available phosphorus (P) in soil samples was determined using the Bray II method, which involves extracting the soil with a solution of 0.03 M NH_4F and 0.1 M HCl. We used a Spectrophotometer UV-Visible (Biochrom Libra S12, LightMachinery Inc., Enabling Science and Industry, Ottawa, Canada). After shaking the soil-extractant mixture for 1 minute, the suspension was filtered, and the phosphorus concentration in the filtrate was measured calorimetrically using the molybdenum blue method. Results were expressed in mg P/kg of dry soil. This method is particularly suitable for acidic soils (Bray and Kurtz, 1945). Exchangeable acidity ($S(Al^{3+} + H^+)$) was measured using Titrimetry by extracting soil samples with 1 M

KCl, followed by titration with NaOH and NaF to determine exchangeable H^+ and Al^{3+}, respectively, according to the method described by (Thomas, 1982).

The saturation of exchangeable bases (SEB) was calculated to assess the proportion of base cations occupying the cation exchange complex of the soil. It was expressed in milliequivalents per 100 grams of soil (meq (100 g)$^{-1}$) using the following formula:

$$\text{SEB } (meq\ (100g)^{-1}) = \left(\frac{Ca^{2+} + Mg^{2+} + K^{+} + Na^{+}\ (meq\ (100g)^{-1})}{\text{CEC } (meq\ (100g)^{-1})}\right) \text{x } 100 \qquad [4]$$

This index reflects the percentage of the cation exchange capacity (CEC) occupied by exchangeable base cations, which are critical for plant nutrition and soil chemical balance. A higher SEB indicates greater availability of essential nutrients and typically corresponds to soils with higher fertility and more neutral pH.

Chapter 7. General discussion

7.1. Overview of the research goals, main results and implications

In this thesis, the functional ecology of *Coffea canephora* in the Congo Basin was investigated to address a central question: how resilient is wild *C. canephora* to climate change across its native range, and through which functional mechanisms is this resilience expressed? This biogeographic region remains understudied despite its critical role in conserving tropical biodiversity and regulating the global climate. Combining century-scale herbarium analyses, a national trait survey of *C. canephora*, and a multi-location altitudinal trial, the study provides convergent evidence that functional leaf traits and stomatal anatomy in *C. canephora* have shifted over time and vary predictably across environmental gradients. Aligning with the conceptual frameworks developed in functional ecology (Violle et al., 2007), this study provides a detailed analysis of the functional responses of this tropical forest understory species to environmental gradients and climate change observed during the 20th and the early 21st centuries.

The results highlight a series of physiological and morphological adjustments that indicate a high degree of functional plasticity, a key component of resilience. They reveal a series of responses consistent with the principles of the leaf economics spectrum (LES) (Wright et al., 2004), highlighting trade-offs between resource acquisition and conservation. LES is widely used to quantify variations in functional traits within plant communities, and facilitate the inferences about species response to climate change over time (Poorter et al., 2009; Verheijen et al., 2013; Reich, 2014). The century-scale shifts observed in specific leaf area (SLA), stomatal traits, and iWUE demonstrate that *C. canephora* has already responded functionally to changing environmental conditions. In particular, the increase in SLA and changes in stomatal morphology presented in Chapter 4 and 5 are consistent with broader studies showing trait adjustments to rising atmospheric CO_2 and warming (Peñuelas and Matamala, 1990; Woodward et al., 2002; Hetherington and Woodward, 2003; Hatangi et al., 2026). These patterns suggest acclimation to altered vapor pressure deficits and potentially higher atmospheric CO_2 (Peñuelas et al., 2011; Van der Sleen et al., 2015; Zhang et al., 2025). These findings align with recent herbarium-based reconstructions that document century-scale shifts in leaf traits and water-use proxies in ecosystems and support the use of historical collections as powerful archives for global-change biology (Bonal et al., 2011; Bauters et al., 2020; Donnelly et al., 2024; Hatangi et al., 2026).

Among other widely addressed adjustments presented in the plant physiological literature, there is increased intrinsic water-use efficiency (iWUE) inferred from $\delta^{13}C$ records during the last century (Bonal et al., 2011; Peñuelas et al., 2011; Van der Sleen et al., 2015; Ma et al., 2021, 2023). In Chapter 5 of this thesis, we found that iWUE of *C. canephora* decreased during the last century, a result inconsistent with general theory, but consistent with two other studies conducted in tropical forests (Huang et al., 2016; Bauters et al., 2020). The observed pattern has been attributed to putative reduced photosynthetic capacity, light limitation or mesophyll conductance. The presented trends explain, in part, the coordinated trait relationships seen in *C. canephora*, notably the negative SLA-iWUE relationship and the positive covariation among stomatal size, density, and maximum diffusive stomatal conductance (g_{cmax})-iWUE (Chapter 5). Patterns of stomatal variation with altitude and climate in this thesis mirror broader ecological patterns reported for tropical and temperate systems (Wang et al., 2014; Liu et al., 2018). Several studies show that stomatal size and density are plastic and often trade off with each other along elevational and climatic gradients, reflecting adjustments in gas-exchange capacity and drought tolerance.

Results of the trial along the altitudinal gradient presented in Chapter 6 corroborate the observed historical patterns. They indicated that growth and functional traits of three genetically distinct varieties varied consistently with both site altitude and geographic origin, demonstrating the species' adaptive plasticity. Traits such as plant height, leaf number, and internode length decreased with elevation, reflecting environmental constraints such as cooler temperatures and reduced resource availability. In contrast, stem diameter, biomass accumulation, and leaf area increased at higher altitudes, highlighting an allocation shift toward structural robustness. The altitudinal responses observed in our multi-location trial, including reduced stomatal size and density at higher elevations, are consistent with both classical observations (Körner et al., 1986) and recent studies reporting decreased stomatal density with increasing elevation and strong environmental regulation of stomatal traits across species and spatial scales (Zhang et al., 2020; Ayala-Ramos et al., 2024). These parallels strengthen the interpretation that the observed changes are functional responses to local microclimate rather than artifacts of sampling. Together, these studies indicate that the trait variation and genotype-by-environment interactions observed in this thesis are ecologically plausible and potentially exploitable for breeding and conservation. Integrating wild germplasm with cultivated gene pools has been proposed as a strategy to buffer coffee cultivation against warming and new pest/disease regimes, an idea that finds support in both trait and genetic studies of

Robusta coffee breeding (Campuzano-Duque et al., 2021; Verleysen et al., 2024).

Correlated changes in traits observed in this study reinforce classical ecological theory on the trade-off between carbon gain and water-use efficiency. Positive correlations between stomatal size, density, and g_{cmax} indicate strategies for maximizing photosynthetic capacity while regulating water loss, whereas negative correlations between SLA and iWUE illustrate resource-conservation trade-offs. These coordinated trait syndromes are consistent with broader patterns reported for tropical understory species and confirm that multiple traits act in concert to mediate resilience under changing climates (Peñuelas et al., 2011; Donnelly et al., 2024).

Genotype-by-environment interactions revealed by multi-site trial underscore the importance of local adaptation. Varieties performed best in environments resembling their native climatic conditions, highlighting evolutionary divergence within the species. Nevertheless, high-altitude sites elicited positive responses in several traits, indicating that lowland-origin *C. canephora* retains plasticity to tolerate cooler conditions. These results suggest that strategic selection of varieties for highland cultivation could buffer coffee production against climate-induced yield losses.

Taken together, our findings indicate that the resilience of *C. canephora* is primarily mediated through trait plasticity, coordinated trait syndromes, and intraspecific diversity. Importantly, this resilience is not uniform across populations but depends on the interaction between genotype and environment, suggesting that adaptive capacity is unevenly distributed across the species' range. This has important implications for the future of the species under climate change: while *C. canephora* shows the capacity to tolerate a range of environmental conditions, its persistence will likely depend on the conservation of its genetic and functional diversity.

From an applied perspective, these results provide a framework for enhancing the resilience of coffee systems. First, they highlight the importance of conserving wild populations across environmental gradients as reservoirs of adaptive traits. Second, they support the targeted selection and deployment of genotypes with favorable functional traits for specific climatic conditions, particularly in emerging highland cultivation zones. Third, they reinforce the potential of integrating wild and cultivated germplasm within agroforestry systems to increase functional diversity and buffer production against climatic variability. Ultimately, leveraging the intrinsic functional diversity of *C. canephora* offers a promising pathway for developing climate-resilient coffee production systems in the Congo Basin and beyond.

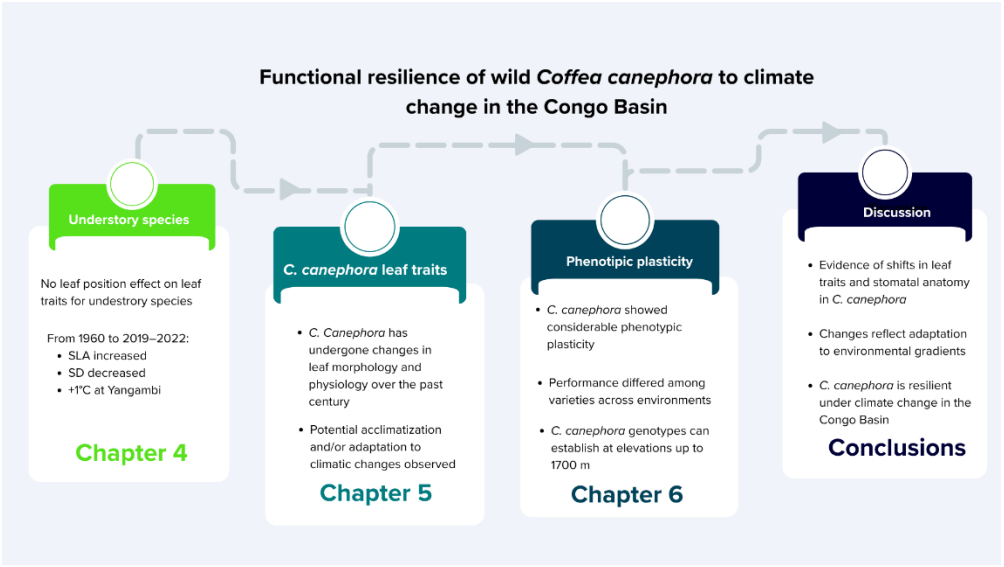


Figure 7-1. Synthesis of the main findings of the thesis on the functional resilience of wild *Coffea canephora* to climate change in the Congo Basin.

7.2. Methodological innovations and challenges

This study follows an innovative approach, combining herbarium-based functional trait research that includes image-based trait extraction, isotopic inference of iWUE, and field validation. Recent methodological studies emphasize how to control for preservation biases and how to interpret isotopic proxies, supporting our analytical choices (Farquhar et al., 1982; Farquhar and Richards, 1984; Ma et al., 2023; Donnelly et al., 2024). While herbarium data are indispensable for detecting long-term trends, they must be interpreted with caution given potential collection biases; complementary field and experimental data, such as those presented here, are essential to validate and contextualize historical signals. From an applied perspective, the combination of trait plasticity and genetic differentiation we found suggests viable adaptation pathways for coffee production under climate change. Several recent reviews and modeling studies highlight the likelihood that Robusta (*C. canephora*) will play an increasingly important role in climate adaptation strategies for global coffee systems because of its greater heat tolerance and broader climatic niche relative to *C. arabica* (Campuzano-Duque et al., 2021; Tournebize et al., 2022; Bollen et al., 2025b). Our results, showing both resilience and limits across altitudinal gradients, provide trait-based mechanistic support for such policy and breeding directions, but they also emphasize the need to conserve wild genetic diversity to provide raw

material for adaptation, consistent to recent studies in the Yangambi region (Depecker et al., 2023; Verleysen et al., 2024). Stomatal traits displayed coordinated adjustments: increased density and pore length, but reduced stomatal size, reflecting the species' ability to maintain gas exchange efficiency under cooler conditions (Wang et al., 2014; Liu et al., 2018). These findings highlight the species' capacity for adaptive plasticity, which likely underpins the observed resilience of wild populations.

Despite the strong contributions of this study, some limitations should be acknowledged. Herbarium specimens, while invaluable for documenting long-term changes, may have been collected in areas that have since experienced significant anthropogenic disturbance. Such land-use changes could influence forest structure and microclimatic conditions and potentially introduce bias when comparing historical and recent samples. Furthermore, herbarium labels often lack detailed information on site-level disturbance histories, such as proximity to roads, logging areas, or agricultural fields. The reliance on modeled historical climate data also introduces uncertainty, as such models may underestimate or overestimate past conditions at fine spatial scales. Similarly, the altitudinal trial, while revealing short-term plastic responses, did not capture multiyear acclimation or reproductive success. Addressing these uncertainties requires longer-term monitoring, incorporation of additional functional traits (roots, hydraulic architecture, reproductive phenology), and integration with genomic analyses to disentangle plasticity from adaptive evolution. Additionally, the trial design did not include *Coffea arabica*, the world's most widely cultivated coffee species and the dominant species at high altitudes. Including *C. arabica* would have enabled direct comparisons between the two species, thereby deepening our understanding of their respective adaptive capacities.

7.3. Future studies

Given these considerations, several avenues of future research emerge. Comparative trials that include both *C. canephora* and *C. arabica* would provide valuable insights into interspecific differences in resilience and adaptation. Expanding trials across a broader range of climatic and geographic conditions would help to validate the generality of the patterns observed here. Moreover, incorporating genomic and transcriptomic analyses could illuminate the molecular mechanisms underlying functional trait variation and adaptive responses. Long-term monitoring of survival, reproductive success, and yield under predicted climate scenarios would further enhance our predictive capacity.

7.4. General conclusions

This thesis addressed the critical challenge of understanding how climate change is impacting the ecology of wild *Coffea canephora*, an understory woody species that plays both an ecological and economic role in the tropical forests of Central Africa. The main objective was to highlight how environmental changes have influenced the functional and physiological traits of *C. canephora*. This research contributes to a broader understanding of plant responses to global climate stressors. It evaluated the species' capacity for resilience and potential for adaptation in novel environments, particularly at higher altitudes in the Congo Basin where climatic conditions differ markedly from traditional lowland habitats, where the species originates and is widely cultivated. Together, these objectives were essential for building a comprehensive framework capable of informing sustainable coffee production strategies under future climate scenarios.

The results generated in this work reveal that climate change has already impacted on functional traits of tropical understory species, with *C. canephora* displaying some of the most pronounced shifts. Through the comparison of pre-1960 herbarium specimens with recent samples collected between 2019 and 2022 in the Yangambi Biosphere Reserve, significant morphological and physiological changes were documented. Specifically, recent leaves were found to be larger and to possess higher specific leaf area, traits that facilitate more efficient light capture under increasingly stressful environmental conditions. At the same time, these recent specimens displayed smaller stomatal pore length and, for most species analyzed, lower stomatal density. Such traits are consistent with adjustments to warmer, drier climates, in which plants must balance carbon acquisition with the need to limit water loss. The fact that these patterns were observed across multiple understory species highlights the pervasive influence of environmental change on tropical forest ecosystems and underscores the value of herbarium collections as long-term ecological archives.

Building on these findings, the spatial and temporal analysis of 179 herbarium specimens of *C. canephora* collected across the DRC provided additional insights into century-scale trait dynamics. Substantial variation was detected in SLA, stomatal size, stomatal pore size, stomatal density, diffusive conductance to CO_2, and stable isotope ratios, as well as intrinsic water-use efficiency. These variations were not random; instead, they reflected coordinated trait–trait relationships that revealed fundamental ecological trade-offs. For example, SLA was negatively associated with intrinsic water-use efficiency, pointing to a clear balance between rapid growth strategies and conservative water-use strategies. Conversely, the positive associations among

stomatal traits and diffusive conductance illustrated how anatomical traits work together to shape physiological capacity. Such integrated trait responses highlight the complexity of plant adaptation processes and provide valuable indicators for predicting how *C. canephora* may respond to ongoing and future climatic pressures.

In addition to the herbarium-based analyses, the experimental component of this research offered a contemporary perspective on *C. canephora*'s adaptive potential. The multi-site trial, conducted across an altitudinal gradient revealed that environmental variation strongly influences growth and functional trait expression. Three genetically distinct varieties were evaluated, and their performance differed markedly depending on both their site of origin and the environmental conditions of the planting sites. Several growth traits, including final plant height, number of leaves, and the distance between nodes, declined with increasing elevation, which is consistent with the cooler temperatures and reduced atmospheric pressure encountered at higher altitudes. In contrast, final stem diameter, leaf area, relative growth rate in leaf area, and aboveground biomass increased, suggesting that plants compensate for reduced vertical growth by investing more in structural strength and leaf surface area. Changes in stomatal traits at higher elevations further emphasize the species' remarkable phenotypic plasticity. Together, these findings confirm that *C. canephora* has the capacity to adjust its morphology and physiology in ways that may enhance survival and productivity under diverse environmental conditions.

The implications of these findings are significant, not only for ecological research but also for the global coffee sector. As climate change continues to reduce suitable coffee-growing regions, particularly at low elevations, the need to develop resilient varieties becomes increasingly urgent. The demonstrated plasticity and adaptive potential of *C. canephora* make it an important candidate for future coffee breeding programs. Moreover, the integration of wild and cultivated *C. canephora* genotypes into high-altitude *C. arabica* plantations may offer an effective strategy for enhancing plantation resilience and reducing the vulnerability of coffee production systems. This research therefore underscores the importance of considering local environmental adaptation and trait expression when planning new cultivation zones or selecting varieties for breeding. For policymakers, these findings emphasize the need to support climate-resilient coffee strategies, including the conservation and use of wild *C. canephora* populations, the promotion of adaptive breeding programs, and the integration of resilient genotypes into existing plantations to secure sustainable production and livelihoods under changing climatic conditions.

In conclusion, the ecological and economic risks associated with climate change, particularly the reduction of suitable coffee-growing areas, demand proactive and coordinated global responses. The evidence presented in this thesis demonstrates that *C. canephora* possesses significant adaptive potential, but also that its long-term persistence and utility depend on targeted and informed management strategies. By grounding future coffee breeding initiatives in robust physiological and functional trait research, the global coffee sector can be better prepared to safeguard production and secure the livelihoods of future generations of coffee producers.

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