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CO_2 ENRICHMENT IN PHOTOAUTOTROPHIC IN VITRO CULTURE OF ‘PÉROLA’ AND ‘TURIPAZ’ PINEAPPLE: IMPACTS ON ACCLIMATIZATION, ANATOMY, AND PHOTOSYNTHESIS

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências Agrárias – PPGIAG/CCA/UEMA, como parte dos requisitos para obtenção do título de Mestre em Ciências Agrárias.

Orientadora: Prof$^{a.}$ Dr^{a}. Thais Roseli Corrêa
Coorientador: Prof. Dr. Sérgio Heitor Sousa Felipe

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Como todo mundo, faço o que posso...
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Summary

Resumo

A micropropagação *in vitro* constitui uma estratégia amplamente utilizada para a multiplicação clonal de plantas; entretanto, os sistemas convencionais de cultivo impõem restrições fisiológicas associadas ao microambiente artificial, que limitam a assimilação de carbono e retardam a aquisição da autotrofia. Nesse contexto, o enriquecimento com CO_2 surge como uma abordagem promissora para mitigar essas limitações, ao induzir ajustes metabólicos, anatômicos e funcionais. Assim, o objetivo deste estudo foi avaliar os efeitos do enriquecimento de CO_2 durante o cultivo *in vitro* fotoautotrófico sobre o crescimento, os atributos relacionados ao carbono e as características micromorfométricas das folhas de *Ananas comosus* das cultivares 'Pérola' e 'Turipaz', bem como verificar como essas respostas se expressam durante a aclimatação *ex vitro*. O experimento foi conduzido em DIC, em esquema fatorial 2 × 2, com duas cultivares ('Pérola' e 'Turipaz') e duas [CO_2], aCO_2 (420 ± 30 µmol mol^{-1}) e eCO_2 (800 ± 30 µmol mol^{-1}), por 50 dias de cultivo *in vitro*, seguidos por 50 dias de aclimatação *ex vitro*. O enriquecimento com CO_2 aumentou significativamente o comprimento da parte aérea e das raízes, o número de folhas, o diâmetro da roseta, a área foliar e a biomassa fresca e seca da parte aérea em ambas as cultivares, enquanto a massa específica foliar não foi afetada. A biomassa radicular apresentou resposta dependente do genótipo, com maiores valores na cultivar 'Turipaz' sob aCO_2, enquanto eCO_2 promoveu incremento da biomassa radicular fresca apenas na cultivar 'Pérola', sem efeito sobre a biomassa radicular seca. O enriquecimento de CO_2 elevou os teores de clorofila *a*, clorofila *b*, clorofila total e carotenoides em ambas as cultivares, com valores de clorofila total consistentemente maiores em 'Turipaz'. O desempenho fotossintético foi favorecido sob eCO_2, com aumentos na *A*, *gs*, *Ci*, *Ci*/*Ca* e *PI*, sem alterações em *A*/*Ci* e *A*/*gs*. O teor de CST aumentou expressivamente sob eCO_2, com valores superiores na cultivar 'Pérola' em ambas as [CO_2]. O enriquecimento de CO_2 promoveu modificações anatômicas foliares, que incluiu aumento da espessura das epidermes, do parênquima aquífero, do parênquima clorofiliano e dos feixes vasculares, com respostas dependentes do tecido e do genótipo sob aCO_2, que foram atenuadas sob eCO_2. Durante a aclimatação *ex vitro*, plantas oriundas do cultivo sob eCO_2 mantiveram maior crescimento, acúmulo de biomassa, teores de pigmentos, carboidratos solúveis totais e parte das características anatômicas avaliadas, com maior vigor geral observado na cultivar 'Turipaz'. Conclui-se que o CO_2 atua como uma variável determinante em sistemas fotoautotróficos de micropropagação do abacaxizeiro, ao modular a trajetória morfofisiológica das plantas durante o cultivo *in vitro* e condicionar positivamente seu desempenho durante a aclimatação *ex vitro*, reforçando a importância de abordagens integradas que considerem a continuidade funcional entre essas duas fases do processo.

Palavras-chave: *Ananas comosus* (L.) Merril; micropropagação; Dióxido de carbono; fotoautotrofia.

Abstract

In vitro micropropagation is a widely used strategy for clonal plant multiplication; however, conventional culture systems impose physiological constraints associated with the artificial microenvironment, which limit carbon assimilation and delay the acquisition of autotrophy. In this context, CO_2 enrichment emerges as a promising approach to mitigate these limitations by inducing metabolic, anatomical, and functional adjustments in plants. Accordingly, the objective of this study was to evaluate the effects of CO_2 enrichment during photoautotrophic *in vitro* culture on growth, carbon-related attributes, and leaf micromorphometric traits of Ananas comosus cultivars 'Pérola' and 'Turipaz', as well as to assess how these responses are expressed during *ex vitro* acclimatization. The experiment was conducted in a completely randomized design in a 2 × 2 factorial arrangement, combining two cultivars ('Pérola' and 'Turipaz') and two [CO_2], aCO_2 420 ± 30 $\mu mol\ mol^{-1}$) and eCO_2 (800 ± 30 $\mu mol\ mol^{-1}$), for 50 days of *in vitro* culture, followed by 50 days of *ex vitro* acclimatization. CO_2 enrichment significantly increased shoot and root length, leaf number, rosette diameter, leaf area, and shoot fresh and dry biomass in both cultivars, while specific leaf mass was not affected. Root biomass exhibited a genotype-dependent response, with higher values in 'Turipaz' under aCO_2, whereas eCO_2 increased root fresh biomass only in 'Pérola', with no effect on root dry biomass. CO_2 enrichment increased chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents in both cultivars, with consistently higher total chlorophyll values in 'Turipaz'. Photosynthetic performance was enhanced under eCO_2, as indicated by increases in net CO_2 A, gs, Ci, Ci/Ca, and PI, without changes in A/Ci or A/gs. TSC content increased markedly under eCO_2, with higher values in 'Pérola' at both [CO_2]. CO_2 enrichment promoted leaf anatomical modifications, including increased thickness of the epidermal layers, aquifer parenchyma, chlorophyll parenchyma, and vascular bundles, with tissue- and genotype-dependent responses under aCO_2 that were attenuated under eCO_2. During *ex vitro* acclimatization, plants derived from eCO_2-grown cultures maintained greater growth, biomass accumulation, pigment contents, total soluble carbohydrates, and part of the evaluated anatomical traits, with overall greater vigor observed in the cultivar 'Turipaz'. In conclusion, CO_2 acts as a key variable in photoautotrophic *in vitro* micropropagation systems for pineapple by modulating plant morphophysiological trajectories during *in vitro* culture and positively conditioning plant performance during *ex vitro* acclimatization, reinforcing the importance of integrated approaches that consider functional continuity between these two phases of the propagation process.

Keywords: *Ananas comosus* (L.) Merril; Micropropagation; Carbon dioxide; Photoautotrophy.

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List of Abbreviations

µg = Microgram
µM = Micromole
A = Net CO_2 assimilation rate
A/Ci = Carboxylation efficiency
A/gs = Intrinsic water use efficiency
ABA = Abscisic acid
Abe = Abaxial epidermis
Ade = Adaxial epidermis
ANA = Naphthaleneacetic acid (NAA)
Ap = Aquiferous parenchyma
BAP = 6-Benzylaminopurine
C3 = C_3 photosynthetic metabolism
CAM = Crassulacean acid metabolism
CST = Total soluble carbohydrates (TSC)
Ci/Ca = Ratio of internal to external CO_2 concentration
cm = Centimeter
cm^2 = Square centimeter
CO_2 = Carbon dioxide
Cp = Chlorenchyma (chlorophyll parenchyma)
CRA = Relative water content (RWC)
DMSO = Dimethyl sulfoxide
E = Transpiration rate
F_0 = Initial fluorescence
Fm = Maximum fluorescence
Fv/Fm = Maximum quantum efficiency of photosystem II
g = Gram
gs = Stomatal conductance
IE = Stomatal index
MF = Fresh mass
mL = Milliliter
mm = Millimeter
GPM = Gas-permeable membranes
DM = Dry mass
MS = Murashige and Skoog medium
TM = Turgid mass
PI = Performance index
PSII = Photosystem II
RC/ABS = Energy absorbed per active reaction center

Chapter I

GENERAL INTRODUCTION AND LITERATURE REVIEW

1. General Introduction

Pineapple (*Ananas comosus var. comusus* L. Merr) is a tropical and subtropical monocotyledonous species belonging to the family Bromeliaceae and the genus *Ananas*, which includes both wild representatives and cultivated types used for economic and ornamental purposes (Collins, 1960; Kessel-Domini et al., 2022). Its center of origin is attributed to South American countries, with Brazil being considered the primary center of genetic diversity for this genus (Silva et al., 2016; Rocha, 2017).

Regarding crop productivity, Brazil ranks fourth worldwide, with an estimated production of 2.4 million tons of pineapple, preceded only by Indonesia (3.1 million tons), the Philippines (2.9 million tons), and Costa Rica (2.9 million tons) (FAOSTAT, 2024). Despite being recognized as one of the centers of origin of *A. comosus* and exhibiting high genetic variability for this species (Souza et al., 2021), more than 95% of commercial pineapple fields in Brazil are composed of only two cultivars, ‘Smooth Cayenne’ and ‘Pérola’ (Reinhardt et al., 2018; Souza et al., 2021).

Among the cultivars commercially grown in Brazil, the ‘Pérola’ cultivar is the most widely produced and the most preferred by consumers when compared to ‘Smooth Cayenne’ (Vieira, 2010). Due to its high economic importance, Pérola’ has been extensively investigated in studies involving biotechnological tools applied to plant propagation (Baptista, 2022; Pires et al., 2023), as well as in studies evaluating substrates during the acclimatization of micropropagated plantlets (Moreira et al., 2006). Additionally, several studies have focused on modifications of the in vitro culture protocol originally proposed by Guerra et al. (1999), including those conducted by Scherer (2008), Kunke et al. (2014), and Paranatinga et al. (2018), as well as studies such as Alves (2021), which evaluated different in vitro cultivation systems.

In addition to ‘Pérola’, other less-explored cultivars exhibit desirable organoleptic characteristics, industrial potential, and commercial appeal, such as the ‘Turiaçu’ cultivar in the state of Maranhão (Reis et al., 2019), which currently ranks third among the most important pineapple cultivars in Brazil.

The ‘Turiaçu’ cultivar originates from the Gurupi microregion, in the state of Maranhão, Brazil, and emerged through local selection, in which indigenous peoples contributed to its domestication and smallholder farmers were responsible for its propagation (Araújo et al., 2007). The ‘Turiaçu’ pineapple shows high acceptance among local consumers due to its sweetness, pleasant aroma, and predominantly more yellowish pulp compared to other cultivars available on the market. However, it presents corky lesions that directly affect the physiological quality of the plant (Araújo et al., 2012; Barboza et al., 2018; Reinhardt et al., 2018; Araujo, 2024).

In addition to the 'Turiaçu' variety, local producers, in collaboration with researchers from UEMA, carried out a natural clonal selection that resulted in the variety known as 'Turipaz'. This variety exhibits superior physical characteristics and lower susceptibility to corky lesions in the fruits compared to the 'Turiaçu' cultivar (Silva et al., 2022a; Silva et al., 2022; Araujo, 2024). However, it maintains similar internal organoleptic characteristics, making it a promising alternative for both fresh consumption and industrial applications.

Despite this potential, the cultivar still occupies a limited cultivation area due to the low availability of planting material. In addition, both 'Turiaçu' and 'Turipaz' are commercially propagated through conventional methods (slips). In this context, studies focusing on biotechnological approaches that support their domestication and propagation under controlled conditions, such as in vitro culture techniques, are essential.

Among the biotechnological tools applied to large-scale plant propagation, tissue culture techniques stand out, particularly *in vitro* propagation, which represents a viable alternative for the large-scale production of disease- and pest-free plantlets (Wijerathna-Yapa et al., 2022).

Tissue culture is recognized as an efficient biotechnological approach for mass plant production; however, several limitations associated with this propagation system still need to be overcome. In general, plants produced under conventional *in vitro* conditions exhibit a mixotrophic metabolism, as they are cultivated in sealed vessels without natural ventilation, leading to the establishment of a microenvironment that compromises plant physiological quality. Plantlets derived from this system exhibit limited morphophysiological performance due to high relative humidity, low CO_2 concentration, and reduced light intensity within the culture environment, factors that collectively restrict gas exchange (Ribeiro et al., 2022).

In light of these limitations, modifications can be implemented to improve the *in vitro* culture system, such as the use of gas-permeable membranes to enhance gas exchange with the external environment, which represents an effective strategy to overcome the constraints of photomixotrophic culture. Saldanha et al. (2012) developed gas-permeable membranes aiming to optimize the natural ventilation of culture vessels and, consequently, improve the gaseous conditions within the *in vitro* system.

Due to their porous nature, these membranes allow aeration within the culture vessels and reduce internal relative humidity, resulting in increased transpiration. Under such conditions, explants can maximize water and nutrient uptake from the culture medium (Xiao et al., 2011; Kozai, 2010).

In addition to the use of gas-permeable membranes, CO_2 enrichment can also be applied as a strategy in plant cultivation. This approach is based on increasing the availability of substrate for photosynthesis, functioning analogously to a form of fertilization. When combined with adequate light conditions and proper management, CO_2 enrichment tends to enhance carbon assimilation, growth, and/or productivity (Xu et al., 2015; Sanches et al., 2017; Villagran et al., 2022; Wang et al., 2022), as well as to induce changes in physiological processes (Wang et al., 2016; Mackinder, 2018), resulting in more robust plants with improved development of the photosynthetic apparatus.

However, studies addressing CO_2 enrichment in plants cultivated in vitro remain scarce, and for pineapple, there are no reports of applying this technique as a fertilization strategy.

In this context, the objective of this study was to evaluate the effects of different CO_2 concentrations under photoautotrophic *in vitro* culture on the morphoanatomical and physiological characteristics, as well as on the acclimatization performance, of the pineapple cultivar 'Pérola' and the variety 'Turipaz'.

2. Literature Review

2.1. Pineapple

Pineapple (*Ananas comosus* var. *comosus* (L.) Merr.) is a perennial monocotyledonous species belonging to the family Bromeliaceae, considered rustic and exhibiting anatomical, morphological, and physiological traits that enhance tolerance to unfavorable conditions, such as water scarcity (Krauss, 1948; Reinhardt et al., 2000). *A. comosus* is native to tropical and subtropical regions of South America, including the South, Southeast, and Central-West regions of Brazil, as well as northern Argentina and Paraguay (Collins, 1960). The dispersion of pineapple across the Americas began through exchanges among indigenous tribes. However, following the discovery of the Americas, the fruit became globally recognized and was introduced into Europe, Asia, and Africa, spreading rapidly across several countries (Ctenas and Quast, 2000; Fouda-Mbanga and Tywabi-Ngeva, 2022).

Globally, approximately one million hectares are dedicated to pineapple production, generating around US$ 9 billion annually for the global economy (Chen et al., 2021; Yabor et al., 2020). In Brazil, pineapple cultivation occupies an area of 62,387 ha, with a production of 1,482,136 tons, corresponding to approximately R$ 2.76 billion in revenue annually. In this context, the North (36%) and Northeast (31%) regions account for the largest shares of national production, with the states of Pará (18%) and Paraíba (13%) being the main contributors (IBGE, 2024).

Among the pineapple cultivars and varieties grown in Brazil, the ‘Pérola’ cultivar stands out due to its economic importance and high export potential, being widely appreciated for fresh consumption and industrial processing (Vieira, 2010), despite its recognized susceptibility to diseases such as fusariosis (Reis et al., 2019).

In the state of Maranhão, pineapple cultivation occupies approximately 2,464 ha, generating around R$ 64.18 million in production value, predominantly distributed across the municipalities of São Domingos do Maranhão (580 ha), Turiaçu (200 ha), Graça Aranha (125 ha), Tuntum (62 ha), and Lago dos Rodrigues (42 ha), with an estimated production of 22,836 tons in 2020 (IBGE, 2023) .

The municipality of Turiaçu, in Maranhão, is particularly noteworthy within the state, as its production is largely concentrated on the ‘Turiaçu’ cultivar, which originated through local selection, involving domestication by indigenous peoples and subsequent propagation by smallholder farmers (Araújo et al., 2007). The ‘Turiaçu’ pineapple plant reaches an average height of approximately 62 cm and produces syncarpic fruits with a cylindrical to conical shape. These fruits have an average weight (without crown) of 1,558 g, length of 20.8 cm, average diameter of 10.4 cm, and central core diameter of 2.5 cm. The crown measures approximately 14.4 cm in length and weighs 61.1 g (Araújo et al., 2012).

The peel and pulp of ripe fruits exhibit a yellow coloration. The total soluble solids (TSS) content averages 16.1 °Brix, with an acidity of 0.38%, resulting in a high sugar-to-acid ratio (42.3). Fruits of this cultivar show strong acceptance among local consumers due to superior attributes compared to other cultivars available on the market, particularly their sweetness, pleasant aroma, and more intense yellow coloration (Reinhardt et al., 2002; Araújo et al., 2012; Kuan et al., 2018; Reis et al., 2023).

The vegetative growth of the ‘Turiaçu’ pineapple variety is influenced by the climatic conditions of its region of origin, the municipality of Turiaçu, Maranhão, and is independent of the fertilization source. It has been observed that the local environmental conditions favor plant development due to water availability, which is often a limiting factor for the expression of the genetic potential of this variety (Ramos et al., 2020).

Producers from the “Serra dos Paz” community in the municipality of Turiaçu, in collaboration with researchers from UEMA, have also selected in recent years four clonal selections derived from the traditional ‘Turiaçu’ pineapple, with particular emphasis on the ‘Turipaz’ variety, which has been empirically compared to ‘Turiaçu’. This variety is characterized by an erect and slender crown, which facilitates harvesting, a more cylindrical fruit shape, wider and flatter fruitlets, and the absence of corky lesions. At the ripe stage, the edges of the fruitlets exhibit a slightly reddish–orange coloration (Dos Reis, 2019; Araujo, 2024).

According to Dos Reis (2029), the ‘Turipaz’ variety has shown tolerance to Fusarium guttiforme, the causal agent of pineapple fusariosis, exhibiting a low level of susceptibility to the pathogen. This disease is considered one of the most important constraints to pineapple production, causing significant losses in yield and fruit quality (Júnior et al., 2023). The ‘Turipaz’ variety presents high commercial potential, with superior physical characteristics compared to ‘Turiaçu’; however, there are no published studies addressing technologies aimed at increasing the production of this variety.

Pineapple is a species that exhibits facultative CAM photosynthetic metabolism, being able to shift between C_3 and CAM pathways in response to environmental conditions. Under favorable conditions, the plant may predominantly operate through C_3 metabolism, whereas under water stress or high radiation, CAM metabolism is induced, characterized by nocturnal stomatal opening and increased water-use efficiency (Zhang et al., 2014; Ming et al., 2015).

Pineapple leaves are arranged in a rosette and display important anatomical adaptations, such as the presence of aquiferous parenchyma for water storage, as well as traits associated with water regulation and photosynthetic performance, including variations in stomatal density and mesophyll structure (Barboza et al., 2006; Alves et al., 2024).

Pineapple propagation is predominantly carried out through conventional vegetative propagules, such as plantlets derived from lateral shoots, commonly referred to as suckers and slips. However, this process is slow, irregular, yields a limited number of propagules, and has a higher potential for the dissemination of pests and diseases. Therefore, the development of techniques that minimize or overcome these limitations has become essential for increasing crop productivity (Barboza and Caldas, 2001; Cardoso, 2014; Couto et al., 2014; Santos et al., 2015; Reinhardt et al., 2018)

In this context, micropropagation has emerged as the biotechnological tool that best meets the demand for large-scale production of healthy, high-quality planting material, as it enables the production of large numbers of clonal plantlets with genetic stability, regardless of seasonality and climatic conditions (George et al., 2008; Almeida, 2025).

2.2.Micropropagation

Plant micropropagation is a biotechnological technique applied to plant tissue culture that enables rapid, large-scale clonal multiplication from different types of explants, such as seeds, shoot apices, nodal segments, adventitious buds, roots, and other plant tissues. This technique is based on cellular totipotency, that is, the ability of plant cells to differentiate and regenerate into a complete and functional organism under controlled *in vitro* conditions, thereby preserving the phenotypic characteristics of the donor plant (George et al., 2008; Hasnain et al., 2022; Murthy et al., 2023).

The micropropagation process involves the isolation, disinfestation, and inoculation of plant cells, tissues, or organs under aseptic conditions, organized into sequential stages including establishment, multiplication, shoot elongation, rooting, and acclimatization to *ex vitro* conditions. The performance at each of these stages depends directly on technical decisions that begin with the appropriate selection of explants and extend to the standardization of aseptic procedures, culture medium composition, the use of plant growth regulators, and environmental conditions during cultivation, such as light intensity and quality, vessel ventilation, and nutrient availability (Almeida et al., 2025; Grzelak et al., 2024).

Compared to seed propagation or conventional vegetative propagation methods, micropropagation stands out for enabling the production of genetically uniform plantlets with high phytosanitary standards and high multiplication rates, which favor batch uniformity and production scaling when associated with automatable systems. In this context, the use of liquid culture systems and bioreactors, including temporary immersion systems, has been widely reported as an efficient alternative to increase productivity and improve the quality of micropropagated plants by optimizing nutrient availability and gas exchange, as well as reducing the limitations commonly associated with semisolid media (Mirzabe et al., 2022; Murthy et al., 2023).

Despite these advantages, the acclimatization of micropropagated plants represents one of the most critical stages of the process, as it involves the transition from a highly controlled environment to *ex vitro* conditions characterized by greater variability in temperature, light intensity, relative humidity, and biotic pressure. Inadequate management at this stage may result in high losses, particularly when the recovery of autotrophic growth and the physiological strengthening of plants are not properly promoted (Grzelak et al., 2024).

2.3. In Vitro Culture – Photomixotrophic and Photoautotrophic Systems

Conventional micropropagation, or photomixotrophic culture, aims primarily to prevent explant contamination; therefore, plants are cultivated in sealed vessels without gas exchange (Ferreira et al., 2021), under conditions of high relative humidity, low light intensity, and with the requirement for exogenous sugars in the culture medium as a source of carbon and energy (Kozai and Kubota, 2001; Barbosa, 2016).

Plant material produced under this system exhibits limited morphophysiological potential, characterized by higher water content in tissues, poorly developed shoots, and leaves that are small, thin, narrow, and underdeveloped. Additionally, these plants show a low density of trichomes and stomata, reduced biomass accumulation, and deficient photoautotrophic activity. These limitations negatively affect plant survival during the *ex vitro* acclimatization process (Wolf et al., 1998; Pospíšilová et al., 1999; Kozai and Kubota, 2001; Xiao and Kozai, 2004; Cha-Um et al., 2011; Xiao et al., 2011; Pinheiro et al., 2021; Ribeiro et al., 2022).

In this context, the use of gas-permeable membranes (GPM) has emerged as an efficient strategy to mitigate the limitations of photomixotrophic culture. In the study conducted by Saldanha et al. (2012), GPMs were developed from polytetrafluoroethylene (PTFE) film combined with layers of microporous tape, specifically designed to enhance natural ventilation in culture vessels.

The use of GPMs in in vitro culture promotes higher photosynthetic rates, increased growth and biomass production, stimulates the development of larger and more robust leaves and roots, improves nutrient uptake, and enhances secondary metabolism (Saldanha et al., 2013, 2014; Ferreira et al., 2019; Ríos-Ríos et al., 2019; Barbosa et al., 2021; Fortini et al., 2021). The increase in gas exchange rates through the use of membranes in photomixotrophic systems reduces the need for exogenous carbon sources in the culture medium, such as sucrose, which in turn improves photochemical efficiency and photosynthetic carbon assimilation (Xiao and Kozai, 2004; Fuentes et al., 2007; Damiani and Schuch, 2008).

The study conducted by Pinheiro et al. (2013) with *Olea europaea* demonstrated that vessel sealing with GPMs enhanced gas exchange within the containers, positively influencing shoot proliferation and promoting improved physiological responses compared to culture without GPMs. Similarly, Soares et al. (2024) reported an increase of 21 $\mu L\ L^{-1}\ s^{-1}$ in CO_2 assimilation with the use of GPMs in the in vitro culture of *Kalanchoe delagoensis* Ecklon & Zeyher. In a study by De Sales et al. (2025), the combination of GPMs with silver nitrate in the *in vitro* culture of *Passiflora edulis* Sims resulted in enhanced plant growth and increased *ex* *vitro* survival, while silver nitrate showed no significant effect on these parameters. Additionally, Vale et al. (2025) reported that the use of GPMs increased the ex vitro survival rate of *Humulus lupulus*.

Recent studies have demonstrated the efficiency of membrane-based systems, such as that reported by Pinheiro et al. (2021) for *Etlingera elatior* (Jack) R.M. Smith (torch ginger), as well as studies on pineapple cv. ‘Pérola’, including those by Pires et al. (2023) and Alves (2021) under photomixotrophic conditions. Additionally, promising results have been reported for the ‘Turiaçu’ variety under photoautotrophic conditions, as described by Alves et al. (2024).

Photoautotrophic culture systems, characterized by the absence of sucrose while maintaining other inorganic nutrients, vitamins, and amino acids, represent an alternative to overcome the limitations of photomixotrophic culture. In this system, it is essential to provide a minimum rate of gas exchange through natural or forced ventilation. This cultivation approach is also referred to as “photosynthetic in vitro culture,” “inorganic culture,” or “sugar-free culture” (Kozai, 1991; Kozai et al., 2005).

Plants derived from photoautotrophic culture exhibit higher photochemical efficiency and increased photosynthetic CO_2 assimilation, resulting in plant material that is metabolically more robust and more tolerant to external environmental factors (Kozai and Kubota, 2001; Xiao and Kozai, 2004; Fuentes et al., 2007; Damiani and Schuch, 2008; Kozai, 2010; Xiao et al., 2011; Batista et al., 2017).

2.4. In Vitro Culture of Pineapple

Studies on the in vitro propagation of pineapple date back to the 1960s (Aghion and Beauchesne, 1960), and since then, research efforts have primarily focused on the development of efficient protocols for the *in vitro* propagation of this fruit crop. The commercial production of plantlets through in vitro culture has significantly expanded over the past two decades, with studies aimed at maximizing both productivity and plant quality. For instance, Mendonça et al. (2017) evaluated the use of organic sources and water-retaining polymers in pineapple plantlets derived from *in vitro* culture, resulting in increased macronutrient accumulation in leaves. Silva et al. (2016) demonstrated that in vitro cultivation of pineapple is feasible under natural light conditions, which directly influences the acclimatization process.

In addition, in vitro culture of pineapple has also been investigated as a strategy for germplasm conservation. Although still limited, studies such as Souza et al. (2018) have reported successful cryopreservation of shoot apices from *Ananas comosus* varieties using droplet vitrification, highlighting the potential for establishing cryobanks of the genus, which may support breeding programs and promote the commercial exploitation of diverse varieties.

In pineapple, *in vitro* propagation has been achieved through different morphogenetic pathways, including organogenesis and somatic embryogenesis. Organogenesis involves the formation of shoots and roots and the *in vitro* regeneration of plantlets, either directly or indirectly. In indirect organogenesis, callus formation is induced in explants, followed by shoot regeneration, whereas in direct organogenesis, shoots or roots arise directly from explant tissues (Phillips and Garda, 2019). Somatic embryogenesis aims to establish a more efficient clonal propagation system based on the balance of plant growth regulators, particularly auxins and cytokinins, in the culture medium, enabling high conversion rates of somatic embryos per explant (Kessel-Domini et al., 2022). Barboza et al. (2006) reported no anatomical differences between micropropagated pineapple plants and those grown under greenhouse conditions, highlighting the efficiency of the technique.

The influence of genotype on *in vitro* development and morphogenetic behavior has been documented for several *Ananas* species, often indicating the need for genotype-specific protocols (Torres et al., 1998). For the ‘Pérola’ cultivar, several studies have adopted the liquid MS medium protocol supplemented with 2.7 µM NAA, 4.4 µM BAP, and 30 g L^{-1} sucrose proposed by Guerra et al. (1999), with modifications aimed at improving outcomes. These include the work of Scherer (2008), who evaluated different exposure times to sodium hypochlorite during disinfestation, Paranatinga et al. (2018), who assessed the effects of BAP concentrations on plant growth and shoot proliferation, and Kunze et al. (2014), who investigated the effects of sucrose concentrations in the culture medium.

Alves et al. (2022) developed an *in vitro* culture protocol for the ‘Turiaçu’ pineapple and further evaluated its photoautotrophic potential and photosynthetic competence, demonstrating that this variety exhibits photoautotrophic capacity under in vitro conditions. Additionally, Silva-Moraes et al. (2024) successfully induced nodular clusters in ‘Turiaçu’, which showed high regenerative potential and efficient microshoot production, indicating a promising approach for large-scale micropropagation of this variety.

Over the past five years, the production of pineapple plantlets via in vitro culture has been further improved through approaches aimed at enhancing protocol efficiency and the morphophysiological quality of plantlets. These include the optimization of light environments using different LED spectra and intensities, resulting in improved morphogenesis and shoot quality (Cavallaro et al., 2023), as well as the refinement of culture media enriched with plant growth regulators to enhance multiplication, shoot formation, and rooting (Lakho et al., 2023). Recent advances also include the use of cultivation systems that facilitate the transition from photomixotrophic to photoautotrophic conditions, combining sucrose modulation with bioreactor systems and gas exchange strategies, leading to improved photochemical efficiency and better acclimatization performance (Alves et al., 2024). Nutritional adjustments have also been shown to enhance plantlet quality and subsequent adaptability (Alavijeh et al., 2025). At a production scale, pilot-scale systems have been developed for micropropagation of commercial varieties, integrating stages from in vitro establishment to rooting and scaling up in temporary immersion bioreactors (Francisco-Rodríguez et al., 2025).

In *in vitro* culture, phytosanitary quality is a central objective, as tissue culture is conducted under aseptic and controlled conditions and is widely used to produce pathogen-free planting material. Reviews on plant tissue culture highlight that these techniques have become essential for producing disease-free plants (Espinosa-Leal et al., 2018). In pineapple, Da Silva et al. (2021) evaluated propagation strategies and the removal of the Pineapple mealybug wilt-associated virus (PMWaV) complex in *in vitro*-grown plants, demonstrating that shoot apex culture is an effective method for virus elimination and for obtaining healthy mother plants, with potential application across different pineapple varieties. Similarly, Guerra et al. (2020) compared shoot apex culture and cryotherapy for the eradication of ampeloviruses associated with wilt, reporting the successful production of virus-free plants and highlighting the potential of these techniques for germplasm sanitation.

At the *ex vitro* stage, recent studies have indicated that improvements *in vitro* conditions can directly influence acclimatization success. For instance, the use of gas-permeable membranes (GPMs) combined with reduced sucrose concentrations has been shown to enhance growth and parameters associated with photosynthetic capacity, thereby improving acclimatization (Alves et al., 2023). Similarly, natural ventilation of culture vessels associated with reduced sucrose levels has been reported to increase gas exchange and promote the development of more functional stomata, resulting in improved acclimatization performance (Pires et al., 2023).

Despite the advances achieved in the micropropagation of the ‘Turiaçu’ variety and the extensive commercial use of the ‘Pérola’ cultivar, there is still a lack of studies focusing on the propagation of the ‘Turipaz’ variety using biotechnological approaches. Moreover, even for ‘Pérola’, gaps remain regarding the optimization of *in vitro* culture protocols, particularly in terms of improving the morphophysiological quality of plantlets. In this context, research exploring biotechnological tools capable of enhancing plant performance during *in vitro* culture, such as photoautotrophic systems, appears promising, as it may contribute to reducing losses during acclimatization and support the large-scale production of more vigorous, uniform, and better-adapted plantlets under *ex vitro* conditions.

2.5. CO_2 Enrichment in Plant Propagation

Atmospheric carbon dioxide (CO_2) plays a fundamental biological role in the formation of organic compounds in chlorophyll-containing plants, constituting the primary substrate for the photosynthetic process (Xu et al., 2015). In general, increased CO_2 availability promotes metabolic changes associated with gas exchange rates and carbohydrate accumulation in plants (Xu et al., 2015; Sanches et al., 2017), which are reflected in physiological modifications such as increased leaf area and crop yield (Wang et al., 2016; Mackinder, 2018; Wang et al., 2020).

Additionally, studies report increases in plant and fruit diameter, weight, and quality under CO_2 enrichment conditions (Dorneles et al., 2019). However, the magnitude of these responses depends directly on interactions with other environmental factors, such as water availability, light intensity, and nutrient supply (Donohue et al., 2013; Dorneles et al., 2019). Moreover, responses may vary among species and according to the type of photosynthetic metabolism (Chakraborty et al., 2008; Ghini et al., 2011).

CO_2 concentration is a key determinant of photosynthesis in C_3 plants, which, under elevated CO_2 conditions, tend to exhibit increased growth and photosynthetic efficiency. In contrast, C_4 plants, which possess specialized anatomical and biochemical mechanisms for carbon fixation, may show comparatively smaller gains in growth and photosynthetic efficiency under elevated CO_2 (Sage and Kubien, 2007; Braga et al., 2021). Recent studies indicate that long-term exposure of C_3 plants to elevated CO_2 may lead to physiological adjustments associated with leaf nitrogen redistribution and a relative reduction in maximum photosynthetic capacity, characterizing processes of carbon acclimation (Bueon et al., 2021; McClain et al., 2023).

Pineapple (*Ananas comosus* (L.) Merr.) is classified as a facultative CAM plant, exhibiting C_3 metabolism under favorable environmental conditions and CAM metabolism under stress conditions such as high temperatures, low relative humidity, high photosynthetically active radiation, and soil water limitation (Taybi et al., 2002; Franco et al., 2014; Taiz and Zeiger, 2017). Recent evidence suggests that, under controlled environments, pineapple may predominantly express C_3 metabolism, especially when adequate light and CO_2

availability are provided, thereby enhancing photosynthetic performance (Alves et al., 2024; Silva-Moraes et al., 2025).

Plant responses to elevated CO_2 are primarily reflected in two key processes: increased photosynthetic rate and reduced stomatal conductance (Ainsworth and Rogers, 2007). These primary responses give rise to additional metabolic effects, including changes in water balance, nitrogen use efficiency, and plant growth. According to Ainsworth and Rogers (2007), such responses are closely associated with the activity of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), although its efficiency depends on factors such as temperature and substrate availability.

Busch and Sage (2017) demonstrated that photosynthetic responses to CO_2 enrichment are not directly related to total Rubisco content, but rather to the capacity for ribulose-1,5-bisphosphate regeneration. Braga et al. (2021) further indicated that this regeneration depends on saturating light conditions and, under elevated CO_2, is modulated by carbohydrate availability, which supplies inorganic phosphate (Pi) for ATP synthesis. Recent studies also indicate that limitations associated with triose phosphate utilization may arise under elevated CO_2, influencing photosynthetic efficiency and biomass accumulation (Gregory et al., 2021; McClain et al., 2023).

Consequently, the enhancement of photosynthetic rates under CO_2 enrichment is more evident in controlled environments, such as growth chambers and greenhouses, where environmental variables can be tightly regulated. In open-field conditions, however, these responses may be altered or attenuated due to climatic variability and interactions with other environmental stresses (Ainsworth and Rogers, 2007; Fu et al., 2024).

CO_2 enrichment also influences the acclimatization phase of plants, as physiological processes may develop compensatory mechanisms that reduce or modulate long-term CO_2 effects (Dorneles et al., 2019). These effects include reductions in the apparent maximum carboxylation rate (Ainsworth and Long, 2005), changes in leaf nitrogen content and Rubisco levels (Braga et al., 2021; Bueon et al., 2021), as well as adjustments in carbon use efficiency.

Plant responses to elevated CO_2 are conditioned by species, environmental conditions, water and nutrient availability, and the predominant photosynthetic metabolism (Braga et al., 2021). Although the literature includes numerous studies on CO_2 enrichment in open systems, such as melon (Araújo et al., 2015), eucalyptus (Machado et al., 2011), coffee (Sanches et al., 2017; De Souza, 2022), and soybean (Pereira-Flores et al., 2013), studies evaluating CO_2 capture and utilization in in vitro systems remain scarce. In this context, recent studies indicate that increasing gas exchange and controlling CO_2 availability *in vitro* can improve photosynthetic activity, growth, and physiological competence, as demonstrated in pineapple (Alves et al., 2024; Silva-Moraes et al., 2025) and other species cultivated under controlled conditions (Pinheiro et al., 2021; Vollmer et al., 2024). However, results remain species- and system-dependent, highlighting the need for species-specific investigations.

Therefore, CO_2 enrichment stands out as a key factor in regulating photosynthesis, plant growth, and acclimatization, particularly in controlled environments. In *in vitro* culture, where limitations in gas exchange and reliance on exogenous carbon sources may compromise plant development, strategies integrating CO_2, light, and nutrition are particularly relevant. For pineapple, a facultative CAM species, the use of photoautotrophic or photomixotrophic systems associated with CO_2 enrichment shows strong potential to improve morphophysiological quality and reduce losses during acclimatization, highlighting the need for targeted studies in this system.

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Chapter II

Photoautotrophic *in vitro* CO_2 enrichment shapes growth of *Ananas comosus* with carryover effects during *ex vitro* acclimatization

Resumo

O enriquecimento de CO_2 em sistemas de cultivo *in vitro* tem sido proposto como uma estratégia para mitigar limitações fisiológicas impostas por ambientes fechados e promover maior eficiência fotossintética em condições fotoautotróficas. Neste estudo, avaliou-se o efeito do enriquecimento de CO_2 durante o cultivo *in vitro* sobre o crescimento, atributos relacionados ao carbono e características micromorfométricas foliares de *Ananas comosus* (cultivares 'Pérola' e 'Turipaz'), bem como sua influência no desempenho das plantas durante a aclimatização *ex vitro*. O experimento foi conduzido em delineamento inteiramente casualizado, em esquema fatorial 2 × 2, com duas concentrações de CO_2 (420 e 800 μmol mol^{-1}). Plantas cultivadas sob CO_2 elevado apresentaram aumento significativo no crescimento, biomassa, área foliar e acúmulo de carboidratos solúveis, além de melhorias nos parâmetros fotossintéticos, incluindo taxa de assimilação de CO_2, condutância estomática e eficiência fotoquímica. Alterações anatômicas consistentes foram observadas, como aumento da espessura do mesófilo, epiderme e tecidos vasculares, bem como redução do índice estomático. Durante a aclimatização *ex vitro*, plantas previamente expostas ao CO_2 elevado mantiveram desempenho superior, com maior crescimento vegetativo, biomassa e conteúdo de pigmentos fotossintéticos. Os resultados demonstram que o enriquecimento de CO_2 atua como um modulador do balanço de carbono e do desenvolvimento estrutural em sistemas fotoautotróficos *in vitro*, com efeitos persistentes que favorecem a competência fisiológica e o estabelecimento *ex vitro* de plantas micropropagadas.

Palavras-chave: enriquecimento de CO_2; micropropagação fotoautotrófica; aclimatização *ex vitro.*

Abstract

CO_2 enrichment in *in vitro* culture systems has been proposed as a strategy to mitigate physiological constraints imposed by closed environments and to enhance photosynthetic performance under photoautotrophic conditions. This study evaluated the effects of CO_2 enrichment during *in vitro* culture on growth, carbon-related attributes, and leaf micromorphometric traits of *Ananas comosus* ('Pérola' and 'Turipaz'), as well as its influence on plant performance during *ex vitro* acclimatization. The experiment was conducted in a completely randomized 2 × 2 factorial design, with two CO_2 concentrations (420 and 800 μmol mol^{-1}). Plants grown under elevated CO_2 exhibited significant increases in growth, biomass accumulation, leaf area, and total soluble carbohydrate content, along with enhanced photosynthetic performance, including higher net CO_2 assimilation, stomatal conductance, and photochemical efficiency. Consistent anatomical modifications were observed, such as increased thickness of mesophyll, epidermis, and vascular tissues, as well as reduced stomatal index. During *ex vitro* acclimatization, plants previously cultured under elevated CO_2 maintained superior performance, with greater vegetative growth, biomass accumulation, and photosynthetic pigment content. These findings demonstrate that CO_2 enrichment acts as a key regulator of carbon balance and structural development in photoautotrophic in vitro systems, with persistent carryover effects that improve physiological competence and *ex vitro* establishment of micropropagated plants.

Keywords: CO_2 enrichment; photoautotrophic micropropagation; *ex vitro* acclimatization

1. Introdution

Pineapple (*Ananas comosus var. comosus* (L.) Merr.) is one of the most important tropical fruit crops worldwide, with high economic and nutritional relevance. Global production exceeds 29 million tons annually, highlighting its role as a major agricultural commodity (Faostat, 2024). However, commercial pineapple propagation is vegetative, and this approach is frequently constrained by recurrent limitations, including low multiplication rates and poor plantlet quality (Cheng et al., 2025; Hossain, 2016).

Micropropagation, or *in vitro* propagation, has been widely adopted as an efficient alternative to conventional vegetative propagation (Murthy et al., 2023). Despite its advantages, conventional *in vitro* culture still imposes physiological limitations on plants, due to the artificial microenvironment established inside culture vessels. Closed containers or systems with low permeability restrict gas exchange, maintain high relative humidity, and promote ethylene accumulation. These conditions limit photosynthetic assimilation and favor mixotrophic or heterotrophic metabolism, sustained by the addition of sucrose to the culture medium (Vollmer et al., 2024; Soares et al., 2024).

Dependence on sucrose as an exogenous carbon source, although necessary for *in vitro* growth, can compromise the acquisition of autotrophy and delay the development of photosynthetic competence in cultured plants (Gago et al., 2022). In addition to metabolic constraints, conventional *in vitro* culture can induce anatomical and physiological imbalances that reduce plant capacity for water regulation and photosynthetic control after transfer to *ex vitro* conditions. As a result, acclimatization is one of the most critical stages of micropropagation (Polivanova et al., 2022; Grzelak et al., 2024).

In this context, CO_2 enrichment has been proposed as a physiological approach in which controlled CO_2 supply, often achieved through injection systems combined with gas permeable membranes, increases gas exchange within culture vessels. Recent studies demonstrate that increased CO_2 availability *in vitro* systems enhance photosynthetic rates, favors the acquisition of photomixotrophy or photoautotrophy, and results in plantlets with improved physiological quality and superior acclimatization performance (Pinheiro et al., 2021; Spinoso-Castillo and Bello-Bello, 2023; Shi et al., 2024). Unlike studies of CO_2 enrichment in ecology or climate change, where CO_2 is a passive environmental factor, in plant tissue culture CO_2 represents an active management variable of the microenvironment. It integrates culture system design and directly influences photosynthetic efficiency, carbon assimilation, and *ex vitro* performance of micropropagated plants.

Studies using CO_2 injection systems or approaches that increase CO_2 availability within the *in vitro* environment have reported higher chlorophyll and carotenoid contents, improved photosynthetic efficiency, and structural modifications, such as increased mesophyll thickness and enhanced differentiation of chlorenchyma. These traits are commonly associated with greater physiological competence of micropropagated plants (Pinheiro et al., 2021; Silva et al., 2024). However, despite these advances, most available studies focus on the *in vitro* phase or assess isolated variables, such as growth, physiology, or anatomy, independently. As a result, integrated approaches linking injected CO_2 enrichment to coordinated anatomical and physiological adjustments and plant performance during *ex vitro* acclimatization remain limited.

Acclimatization is widely recognized as one of the main technical and biological bottlenecks of micropropagation because *ex vitro* conditions impose physiological and structural adjustments that often result in plant losses and reduced vigor (Grzelak et al., 2024). Evidence indicates that morphological, anatomical, and physiological traits are expressed during *ex vitro* acclimatization are established during the *in vitro* phase in response to microenvironmental and culture system management, thereby conditioning plant capacity for post-transplant adjustment (Xiao et al., 2011; Silva et al., 2024).

Nevertheless, despite advances in micropropagation systems, most studies remain restricted to evaluations conducted at a single phase of the process or focused on isolated traits (Bello-Bello et al., 2025). Consequently, integrated quantitative frameworks connecting *in vitro* culture conditions with morphological, physiological, and structural performance throughout *ex vitro* acclimatization remain scarce, and these relationships are often inferred indirectly or treated only in a contextual manner (Soares et al., 2024).

Based on this context, we hypothesized that CO_2 enrichment during *in vitro* culture modulates growth, carbon balance, and leaf anatomical traits of *Ananas comosus*, and that these *in vitro*-induced responses influence plant performance during the subsequent *ex vitro* acclimatization phase. Accordingly, the objective of this study was to evaluate the effects of CO_2 enrichment during *in vitro* culture on growth, carbon-related attributes, and leaf micromorphometric traits of *Ananas comosus*, and to assess how these responses expressed during *ex vitro* acclimatization.

2. Materials and Methods

2.1. Plant material and *in vitro* culture conditions

The experiment was conducted at the Plant Tissue Culture Laboratory (LCT) of the State University of Maranhão (UEMA), Maranhão, Brazil. Shoots of the pineapple cultivars 'Pérola' and 'Turipaz', maintained *in vitro* at LCT/UEMA, were used. This bank is maintained in 350 mL glass flasks containing 30 mL of semisolid Murashige and Skoog (MS) medium with vitamins (Murashige and Skoog, 1962) (PhytoTechnology®, Lenexa, KS, USA), supplemented with 200 mg L^{-1} myo-inositol (Sigma-Aldrich®, St. Louis, MO, USA), 4 μM 6-benzylaminopurine (BAP) (Sigma-Aldrich®, St. Louis, MO, USA), 2 μM naphthaleneacetic acid (NAA) (Sigma-Aldrich®, St. Louis, MO, USA), 30 g L^{-1} sucrose (Dinâmica® Ltda, Jardim da Glória, SP, Brazil), and 2 g L^{-1} phytagel (Sigma-Aldrich®, St. Louis, MO, USA).

The pH of the culture medium was adjusted to 5.7 ± 0.03 prior to autoclaving at 121 °C and 1.5 atm for 15 min. Cultures were maintained at 25 ± 2 °C under an irradiance of 100 μmol m^{-2} s^{-1} provided by four white tubular LED lamps (T8, 9 W; Avant, São Paulo, SP, Brazil), with a 16 h photoperiod.

For the experimental setup, four shoots of both pineapple cultivars, approximately 1.5 cm in length, were transferred to 350 mL glass flasks containing 60 mL of MS medium (PhytoTechnology®, Lenexa, KS, USA) supplemented with vitamins, 200 mg L^{-1} myo-inositol, and 2.7 μmol L^{-1} NAA, and solidified with 5.5 g L^{-1} agar (Agargel®, João Pessoa, Brazil). The sucrose-free medium (0 g L^{-1}) was used to establish a photoautotrophic cultivation system. The pH was adjusted to 5.70 ± 0.03, and the medium was autoclaved at 121 °C and 1.5 atm for 15 min. The flasks were sealed with polypropylene caps containing two 10 mm diameter holes covered with microporous tape membranes (Fig. 1A), following the method proposed by Saldanha et al. (2012).

2.2. CO_2 enrichment in growth chambers

Two growth chambers (Model TE-4002/3, Tecnal®, Piracicaba, São Paulo, Brazil) were used, each with internal dimensions of 1500 mm (height) × 1000 mm (width) × 800 mm (depth). Environmental conditions inside the chambers were maintained at 25 °C, with relative humidity of 60% and a photosynthetic photon flux density (PPFD) of 100 μmol m^{-2} s^{-1}, provided by SG Delta LED modules emitting blue, red, and white spectra. Air circulation was ensured by a continuous forced-air ventilation system operating at a flow rate of 500 m^3 h^{-1} at 0 Pa (Fig. 1 B).

The chamber atmosphere was subjected to CO_2 enrichment, with either ambient CO_2 (aCO_2, 420 ± 30 μmol mol^{-1}) or elevated CO_2 (eCO_2, 800 ± 30 μmol mol^{-1}) supplied from a compressed CO_2 cylinder. A 16 h photoperiod was established within the chambers, and plants were maintained under these controlled conditions for 50 days.

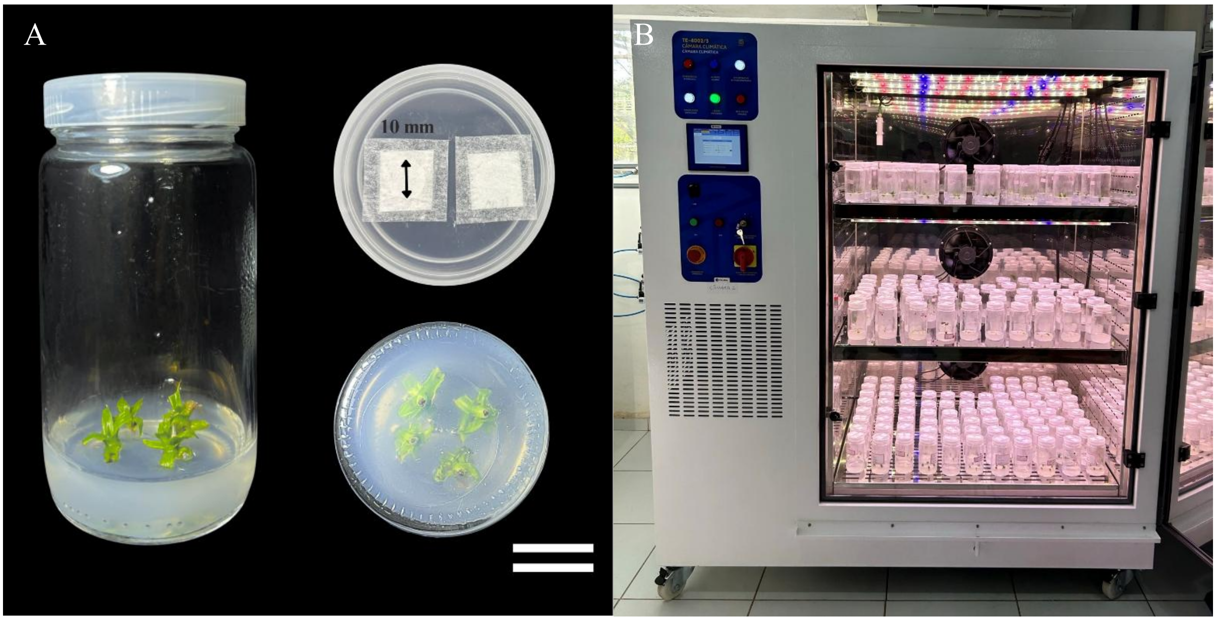

Fig 1. Experimental setup for photoautotrophic *in vitro* culture and CO_2 enrichment. (A) Glass culture vessels containing four pineapple shoots grown in MS medium andsealed with polypropylene lids containing gas-permeable membranes (10 mm diameter) to promote gas exchange; (B) Growth chamber used for CO_2 enrichment, equipped with LED lighting and a forced-air circulation system, where cultures were maintained under controlled environmental conditions. Scale bar: 2 cm.

2.3.Growth analysis

Growth parameters evaluated included shoot length (cm), rosette diameter (cm), length of the longest root (cm), number of leaves, shoot fresh mass (g), shoot dry mass (g), root fresh mass (g), root dry mass (g), and specific leaf mass (g cm^{-2}). Leaf area (cm^2) was determined from digital images using ImageJ software (National Institutes of Health, Bethesda, MD, USA), version 1.49v. Shoots and roots were dried in a forced-air oven at 60 °C until constant mass.

2.4.Chlorophyll a fluorescence

Basal fluorescence (Fo), maximum fluorescence (Fm), variable fluorescence (Fv), maximum quantum efficiency of PSII (Fv/Fm), the Fm/Fo ratio, the RC/ABS ratio, and the performance index (PI) were determined after 30 min of dark adaptation. Dark adaptation ensured that all PSII reaction centers were fully open, minimizing non-photochemical energy dissipation.

Measurements were performed on the fourth fully expanded leaf, counted from the innermost part of the rosette outward, using a non-modulated chlorophyll fluorometer (Pocket PEA, Plant Efficiency Analyzer; Hansatech, King's Lynn, UK), following the protocol described by Gonçalves et al. (2010).

2.5. Leaf gas exchange

Leaf gas exchange measurements were performed under saturating artificial light (1,000 μmol m^{-2} s^{-1}) on the fourth fully expanded leaf. External CO_2 concentration was maintained at either ambient (aCO_2, 420 μmol mol^{-1}) or elevated (eCO_2, 800 μmol mol^{-1}) levels, according to each treatment, with an average air temperature of 30 °C. Gas exchange parameters were recorded using an open gas exchange system (LI-6400XT, LI-COR Biosciences, Lincoln, NE, USA), including net CO_2 assimilation rate (A), stomatal conductance to water vapor (gs), transpiration rate (E), intercellular CO_2 concentration (Ci), the Ci/Ca ratio, carboxylation efficiency (A/Ci), and intrinsic water-use efficiency (A/gs).

2.6. Determination of total soluble carbohydrates

Total soluble carbohydrates were determined using leaf samples previously dried at 60 °C. Extracts were prepared from 0.020 g of finely macerated plant material mixed with 4 mL of distilled water. Samples were homogenized using a vortex mixer and centrifuged twice, first at 3,000 rpm for 15 min and subsequently at 6,000 rpm for 10 min, both at room temperature. The supernatant was collected after each centrifugation step.

For the colorimetric assay, 100 μL of the extract was transferred to test tubes in triplicate, followed by the addition of 100 μL of distilled water, 500 μL of 5% phenol, and 2.5 mL of concentrated sulfuric acid. Tubes were placed in an ice-water bath at room temperature (25 °C) for 10–20 min to allow cooling and color stabilization and were then vortexed for 20 s.

Absorbance was measured at 490 nm using a UV–visible single-beam spectrophotometer (UV-M51; BEL Engineering Company, Monza, Italy). The blank consisted of 500 μL of distilled water, 500 μL of 5% phenol, and 2.5 mL of concentrated sulfuric acid. Total soluble carbohydrate concentration was calculated based on a standard curve following the method described by Dubois et al. (1956).

2.7. Quantification of photosynthetic pigments

Three leaf discs (3 mm diameter) were collected from the fourth fully expanded leaf, counted from the innermost part of the rosette outward. Discs were immersed in 3 mL of dimethyl sulfoxide (DMSO; Isofar® Ltda., Duque de Caxias, RJ, Brazil) and maintained in the dark for 48 h, following the procedure described by Santos et al. (2008).

Absorbance was measured at 665 nm, 649 nm, and 480 nm, using a UV–visible spectrophotometer (UV-M51; BEL Engineering Company, Monza, Italy) equipped with a quartz cuvette with a 10 mm optical path length. Chlorophyll a, chlorophyll b, and carotenoid contents were calculated according to Wellburn (1994).

2.8.Stomatal index

To evaluate the stomatal index, epidermal impressions were obtained from the abaxial surface of the fifth fully expanded leaf, counted from the innermost part of the rosette outward, using instant adhesive (SuperBonder®). Impressions were examined under a light microscope (model B20T; Bioptika, Colombo, PR, Brazil) equipped with a U-photo system and a digital camera (model CMOS-5.0; Bioptika, Colombo, PR, Brazil). Images were analyzed using ImageJ software, and the stomatal index was calculated according to Cutter (1968).

2.9. Relative leaf water content

Relative leaf water content (RWC) was determined following the method of Barr and Weatherley (1962), with modifications. Five leaf discs (3 mm diameter) were collected from the fourth fully expanded leaf of each plant, counted from the innermost part of the rosette outward. Discs were immediately weighed to obtain fresh mass (FM) and then immersed in approximately 20 mL of distilled water in plastic containers for 24 h. After rehydration, discs were weighed to determine turgid mass (TM). Subsequently, discs were dried at 60 °C to obtain dry mass (DM).

Relative water content was calculated as:

$$\mathrm{RWC}\ (\%) = [(\mathrm{FM} - \mathrm{DM}) / (\mathrm{TM} - \mathrm{DM})] \times 100$$

2.10. Leaf anatomy and micromorphometric analysis

Samples from the fifth fully expanded leaf, counted from the innermost part of the rosette outward, were collected between 8:00 and 10:00 a.m. (Brasília time, UTC−3) and fixed in FAA50 (formaldehyde: acetic acid: 50% ethanol, 1:1:18, v/v/v) for 48 h. Samples were dehydrated in a graded ethanol series (30–100%) and embedded in Historesin® (Leica

Instruments, Jena, Germany). Cross-sections with a thickness of 7 µm were obtained using a rotary microtome (Lupetec® MRP2015, São Carlos, Brazil) and stained with 0.05% toluidine blue (pH 4.0), following O'Brien and McCully (1981).

Micromorphometric analyses were performed using digital images acquired at 10× magnification. Measurements of cell dimensions in the abaxial and adaxial epidermis, palisade parenchyma, mesophyll, and intercellular spaces were obtained in both width and height, with intercellular spaces quantified based on area. All measurements were performed using ImageJ software.

For each treatment, five biological replicates were analyzed. For each biological replicate, three technical replicates were obtained from histological sections at different depths. Technical replicates were averaged to generate a single value per biological replicate, which was used for statistical analysis.

2.11. Plant acclimation

Plants grown in the growth chambers were removed from the flasks, and their roots were washed under running water to remove residual culture medium. Subsequently, the plants were transplanted into 200 mL plastic cups containing Carolina Soil® substrate and transferred to a greenhouse maintained at a temperature of 30 ± 2 °C and relative humidity of approximately 80%. Plants were maintained under these conditions for 50 days. After this period, the analyses described above were performed again.

2.12. Statistical analysis

The experiment followed a completely randomized design in a 2 × 2 factorial arrangement, consisting of two pineapple cultivars ('Pérola' and 'Turipaz') and two atmospheric CO_2 concentrations (420 and 800 ± 30 µmol mol^{-1}), with twenty replications per treatment. Each experimental unit consisted of one flask containing four shoots. Data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Bartlett's test.

Leaf area, shoot dry mass, stomatal conductance (gs), intercellular CO_2 concentration (Ci), the Ci/Ca ratio, and carboxylation efficiency (A/Ci) under *in vitro* conditions, as well as leaf area and rosette diameter under *ex vitro* conditions, were log-transformed [log(x + 1)] to meet the assumptions of analysis of variance (ANOVA). These data were subsequently subjected to ANOVA, and mean comparisons were performed using Tukey's test at a 5% significance level.

Variables that did not meet normality assumptions even after transformation, including root length, root fresh mass, root dry mass (*in vitro*), rosette diameter, maximum quantum efficiency of PSII (Fv/Fm), and total soluble carbohydrates (*ex vitro*), were analyzed using generalized linear models (GLMs) with a Gamma distribution and a log link function (Mora et al. 2008). Adjusted means were estimated using the *emmeans* package, and multiple comparisons were performed using Scheffé's post hoc test with adjustment for multiple comparisons (adjust = "scheffe"). All statistical analyses were performed using R software (*version 4.5.1*; R Core Team, 2025).

3. Results

3.1. *In vitro* morphological performance, growth, and biomass of plants

After 50 days of *in vitro* cultivation, pineapple plants of the cultivars 'Pérola' and 'Turipaz' exhibited visible phenotypic differences between the aCO_2 and eCO_2 treatments. Under aCO_2, plants of both cultivars showed reduced vegetative development, with narrower leaves and a less developed root system. In contrast, under eCO_2, plants displayed greater shoot and root development, with a higher number of shoots and more conspicuous roots (Fig. 2).

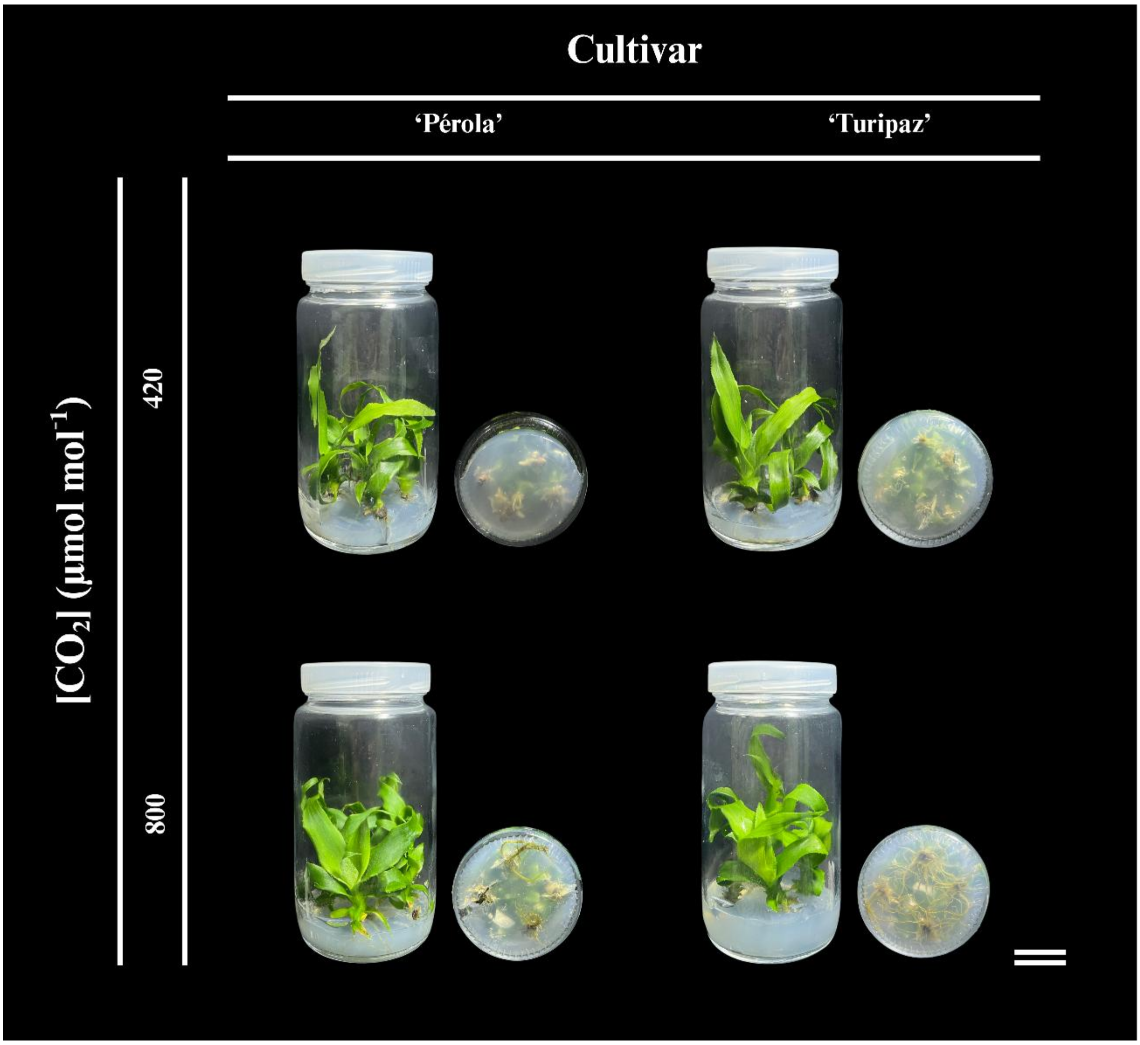

Fig 2. Phenotypic responses of *in vitro*-grown *Ananas comosus* plants of the cultivars 'Pérola' and 'Turipaz' under two CO_2 concentrations (420 and 800 µmol mol^{-1}) after 50 days of cultivation. Scale bar = 2 cm.

Shoot length increased significantly under eCO_2 in both cultivars, with increments of 2.0 cm in 'Pérola' and 1.3 cm in 'Turipaz' compared with aCO_2 (Fig. 3A). Root length was also significantly enhanced by eCO_2 in both cultivars, increasing by 1.9 cm in 'Pérola' and 2.7 cm in 'Turipaz' (Fig. 3B). Under aCO_2, 'Pérola' exhibited greater root length than 'Turipaz', whereas under eCO_2, higher values were observed for 'Turipaz' (Fig. 3B).

The number of leaves increased significantly under eCO_2 in both cultivars (Fig. 3C). Rosette diameter was also significantly greater under eCO_2, whereas no differences between cultivars were detected (Fig. 3D).

Shoot fresh biomass was significantly higher under eCO_2 in both cultivars, with increases of 0.35 g in 'Pérola' and 0.38 g in 'Turipaz' relative to aCO_2 (Fig. 3E). For root fresh biomass, 'Turipaz' exhibited higher values than 'Pérola' under aCO_2. Under eCO_2, root fresh biomass increased significantly in ‘Pérola’, whereas no CO_2 effect was detected for ‘Turipaz’ (Fig. 3F).

Shoot dry biomass was higher under eCO_2, increasing from 0.163 to 0.403 g across cultivars (Fig. 3G). For root dry biomass, ‘Turipaz’ showed higher values than ‘Pérola’ under aCO_2, however, this difference was not maintained under eCO_2, and no CO_2 effect was detected for this variable (Fig. 3H).

Leaf area increased significantly under eCO_2 in both cultivars (Fig. 3I). Specific leaf mass was not affected by CO_2 concentration, although ‘Pérola’ exhibited consistently higher values than ‘Turipaz’ across treatments (Fig. 3J). Stomatal index was reduced under eCO_2 in both cultivars, with a mean reduction of 59% relative to aCO_2 (Fig. 3K).

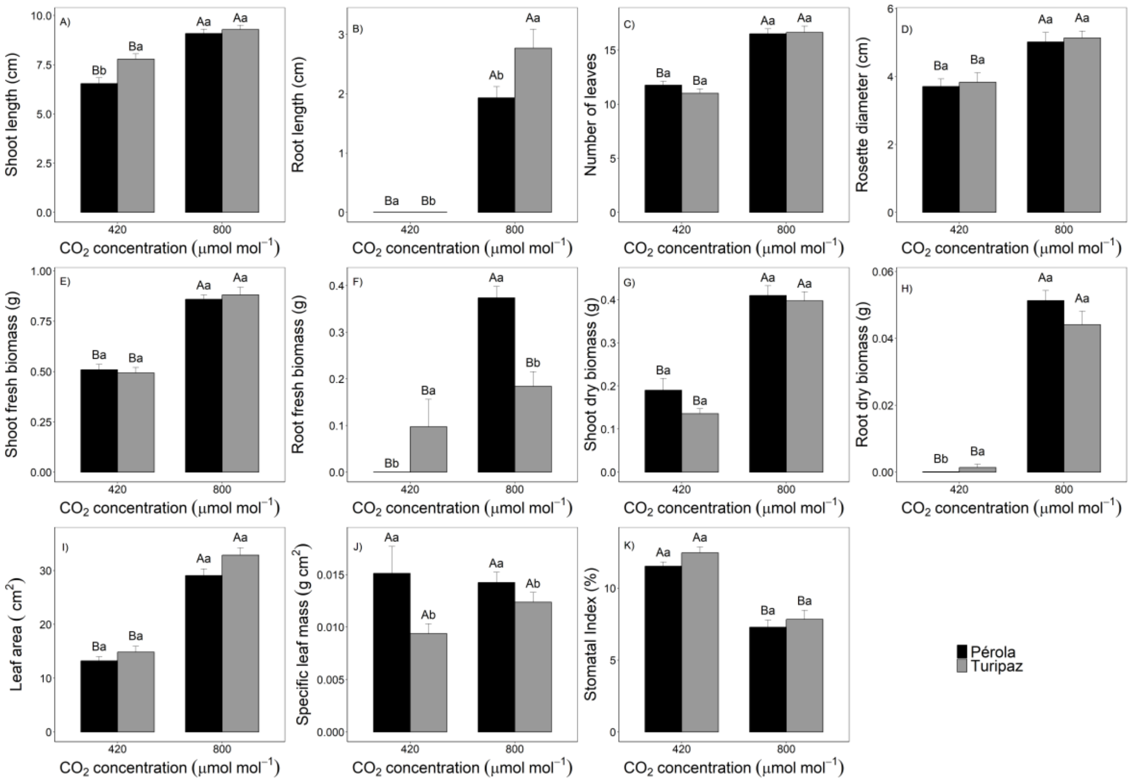

Fig 3. Growth, biomass accumulation, and stomatal index of *in vitro*-grown *Ananas comosus* plants after 50 days under two CO_2 concentrations (420 and 800 μmol mol⁻¹). (A) Shoot length*; (B) Root length; (C) Number of leaves; (D) Rosette diameter; (E) Shoot fresh* biomass; (F) Root fresh biomass; (G) Shoot dry biomass; (H) Root dry biomass; (I) Leaf area; (J) Specific leaf mass; and (K) Stomatal index. Uppercase letters indicate significant differences between CO_2 concentrations within the same cultivar, whereas lowercase letters indicate significant differences between cultivars within the same CO_2 concentration (Tukey test, $P \leq 0.05$). Values represent means ± standard error (n = 8).

3.2.*In vitro* photosynthetic pigment composition under aCO_2 and eCO_2

Chlorophyll a content increased significantly under eCO_2 in both cultivars, with absolute increases of 22.3 µg cm^{-2} in 'Turipaz' and 13.5 µg cm^{-2} in 'Pérola' compared with plants grown under aCO_2 (Fig. 4A). A similar response was observed for chlorophyll b, which was significantly higher under eCO_2 in both cultivars (Fig. 3B). Accordingly, the chlorophyll a/b ratio differed between CO_2 treatments, with higher values under eCO_2 and no significant differences between cultivars (Fig. 4C).

Total chlorophyll content was significantly enhanced under eCO_2, with higher values observed in 'Turipaz' than in 'Pérola' across CO_2 treatments (Fig. 4D). Carotenoid content also increased significantly under eCO_2 in both cultivars, whereas no significant differences between cultivars were detected (Fig. 4E). In contrast, the total chlorophyll-to-carotenoid ratio was not significantly affected by CO_2 concentration or cultivar (Fig. 4F).

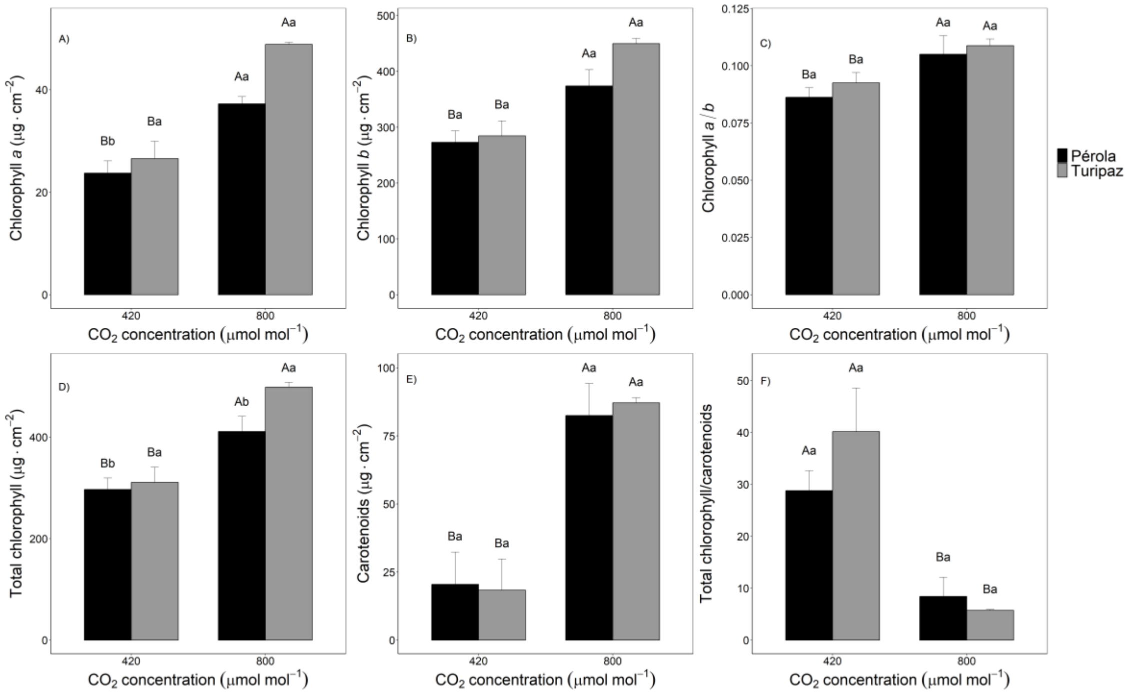

Fig 4. Photosynthetic pigment contents of *in vitro*-grown *Ananas comosus* plants after 50 days under two CO_2 concentrations (420 and 800 µmol mol^{-1}). (A) Chlorophyll a; (B) Chlorophyll b; (C) Chlorophyll a/b ratio; (D) Total chlorophyll; (E) Carotenoids; and (F) Total chlorophyll-to-carotenoid ratio. Uppercase letters indicate significant differences between CO_2 concentrations within the same cultivar, whereas lowercase letters indicate significant differences between cultivars within the same CO_2 concentration (Tukey test, $P \leq 0.05$). Values represent means ± standard error (n = 8).

3.3. *In vitro* chlorophyll fluorescence and gas exchange under aCO_2 and eCO_2

Significant cultivar-dependent differences were observed for Fv/Fm, with ‘Turipaz’ showing consistently higher values than ‘Pérola’ under both aCO_2 and eCO_2, with absolute differences of 0.011 and 0.020, respectively (Fig. 5A). A similar response pattern was observed for RC/ABS, with higher values in ‘Turipaz’ than in ‘Pérola’ under aCO_2, and an additional increase under eCO_2 in both cultivars (Fig. 5B).

The performance index (PI) differed between CO_2 concentrations, with higher values under eCO_2 in both cultivars. Under eCO_2, PI increased by 1.19 units in ‘Pérola’ and by 1.54 units in ‘Turipaz’. Across both CO_2 levels, ‘Turipaz’ consistently exhibited higher PI values than ‘Pérola’ (Fig. 5C).

Gas exchange parameters, including net photosynthetic rate (A), stomatal conductance (gs), intercellular CO_2 concentration (Ci), and the Ci/Ca ratio, showed higher values under eCO_2. Mean increases under eCO_2 were 5.6 μmol m^{-2} s^{-1} for A, 0.10 mmol H_2O m^{-2} s^{-1} for gs, 322 μmol mol^{-1} for Ci, and 0.20 for Ci/Ca, with higher values generally observed in ‘Pérola’ (Fig. 5D–F, H).

Transpiration rate (E) also increased under eCO_2, with a mean increase of 3.11 mmol H_2O m^{-2} s^{-1}, while higher values were observed in ‘Turipaz’ across CO_2 treatments (Fig. 5G).

In contrast, intrinsic carboxylation efficiency (A/Ci) and intrinsic water-use efficiency (A/gs) were not affected by CO_2 concentration, with values ranging from 0.0177 to 0.0239 for A/Ci and from 98.2 to 161 for A/gs (Fig. 5I, J).

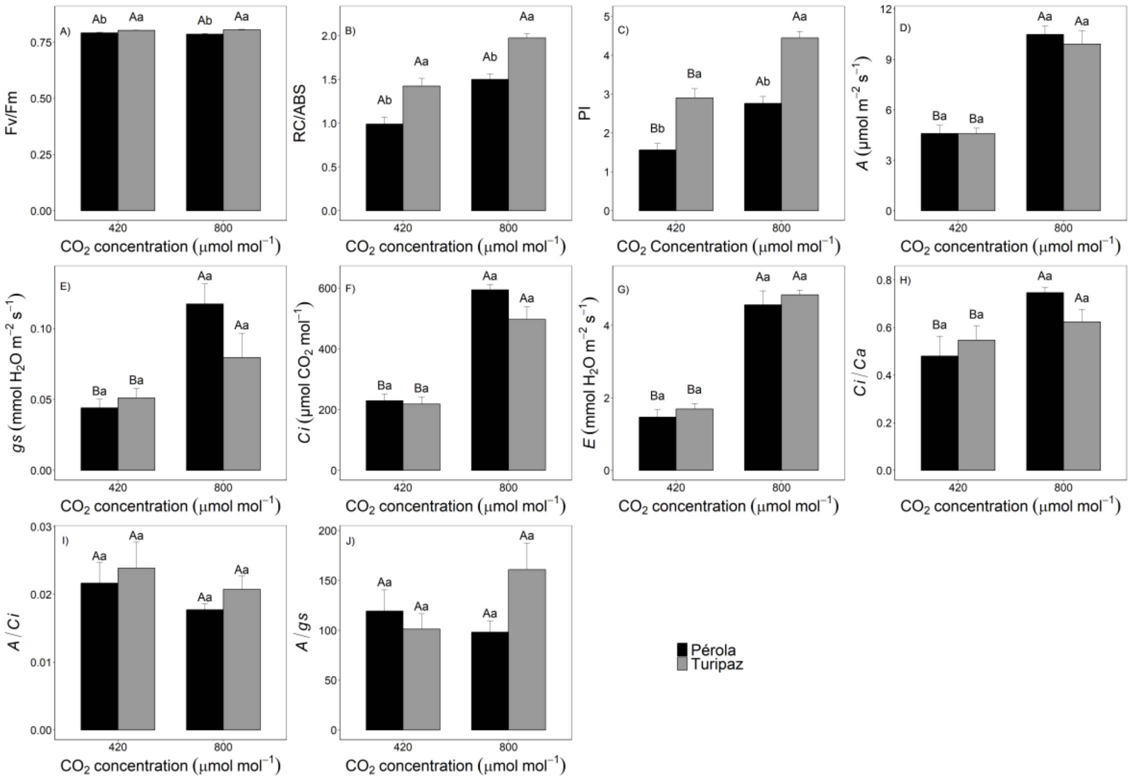

Fig 5. Chlorophyll fluorescence and gas exchange parameters of *Ananas comosus* plants after 50 days of *in vitro* culture under two [CO_2] levels (420 and 800 ± 30 µmol mol^{-1}). (A) Fv/Fm; (B) RC/ABS; (C) PI; (D) net photosynthetic rate (A, µmol m^{-2} s^{-1}); (E) stomatal conductance (gs, mmol H_2O m^{-2} s^{-1}); (F) intercellular CO_2 concentration (Ci, µmol CO_2 mol^{-1}); (G) transpiration rate (E, mmol H_2O m^{-2} s^{-1}); (H) Ci/Ca; (I) A/Ci; (J) A/gs. Uppercase letters indicate significant differences between [CO_2] levels within the same cultivar, whereas lowercase letters indicate significant differences between cultivars within the same [CO_2] level (Tukey test, $P \leq 0.05$). Values represent means ± standard error (n = 8).

3.4.*In vitro* soluble carbohydrate accumulation under aCO_2 and eCO_2

Total soluble carbohydrate content was higher under eCO_2 than under aCO_2, with a mean increase of 654 mg g^{-1} DW, corresponding to a 183% increase relative to aCO_2 (Fig. 6). Across both CO_2 concentrations, the cultivar ‘Pérola’ consistently exhibited higher total soluble carbohydrate values than ‘Turipaz’.

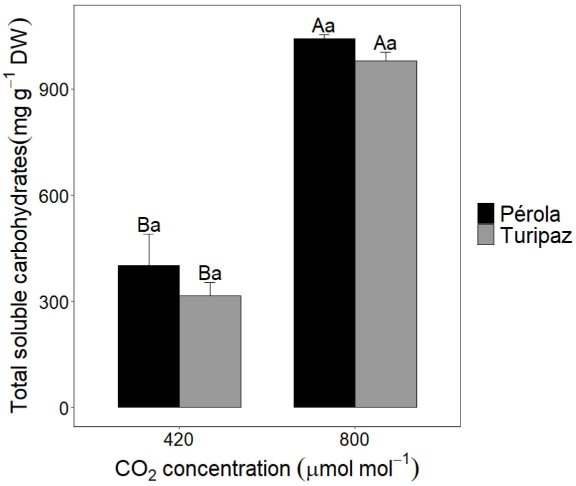

Fig 6. Total soluble carbohydrate content in *Ananas comosus* plants after 50 days of *in vitro* culture under two [CO_2] levels (420 and 800 ± 30 μmol mol^{-1}). Uppercase letters indicate significant differences between [CO_2] levels within the same cultivar, whereas lowercase letters indicate significant differences between cultivars within the same [CO_2] level (Tukey test, $P \leq 0.05$). Values represent means ± standard error (n = 8).

3.5. *In vitro* leaf micromorphometric traits under aCO_2 and eCO_2

Under ambient aCO_2 conditions, both cultivars exhibited more compact tissues, with relatively smaller cells in the chlorenchyma and aquiferous parenchyma. In contrast, plants grown under elevated eCO_2 showed a tendency toward increased cell expansion, particularly in the aquiferous parenchyma, as well as a more loosely organized mesophyll structure (Ig. 7A).

Differences between cultivars were also evident. The ‘Turipaz’ variety exhibited a more homogeneous tissue organization and greater cell expansion under elevated CO_2, while ‘Pérola’ showed more discrete anatomical changes between treatments. The vascular bundles remained structurally preserved across treatments (Fig. 7A).

Abaxial epidermis thickness differed between CO_2 concentrations, with higher values observed under eCO_2 in both cultivars (Fig. 7B). Adaxial epidermis thickness also showed higher values under eCO_2. Under aCO_2, ‘Turipaz’ exhibited higher adaxial epidermis thickness than ‘Pérola’, whereas no differences between cultivars were detected under eCO_2 (Fig. 7C).

Aquifer parenchyma thickness was higher under eCO_2 in both cultivars, with no differences detected between genotypes within each CO_2 level (Fig. 7D). Chlorophyll parenchyma thickness differed between cultivars under aCO_2, with higher values in ‘Turipaz’, while under eCO_2 both cultivars exhibited similar thickness values (Fig. 7E).

Transverse vascular bundle thickness showed higher values under eCO_2 in both cultivars, without differences between genotypes within each CO_2 concentration (Fig. 7F). Longitudinal vascular bundle thickness did not differ between CO₂ concentrations in ‘Pérola’. In contrast, ‘Turipaz’ exhibited higher longitudinal vascular bundle thickness under eCO₂, although no differences between cultivars were detected within each CO₂ level (Fig. 7G).

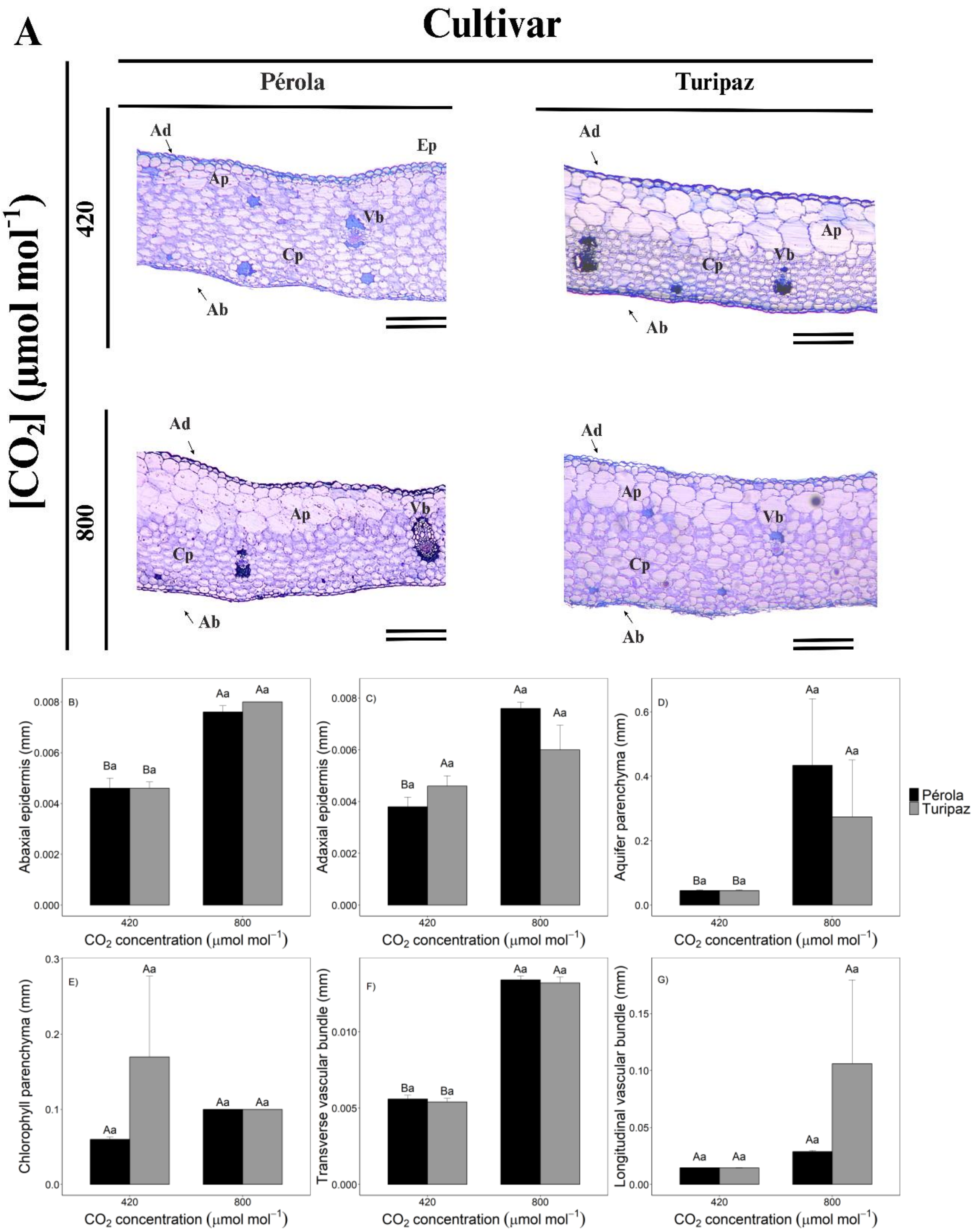

Fig 7. Leaf micromorphometric parameters of *Ananas comosus* plants after 50 days of *in vitro* culture under two [CO_2] levels (420 and 800 ± 30 μmol mol^{-1}). (A) Leaf cross section (scale bar = 200μm); (B) Abaxial epidermis thickness (mm); (C) adaxial epidermis thickness (mm); (F) transverse vascular bundle thickness (mm); and (G) longitudinal vascular bundle thickness (mm). Uppercase letters indicate significant differences between [CO_2] levels within the same cultivar, whereas lowercase letters indicate significant differences between cultivars within the same [CO_2] level (Tukey test, $P \leq 0.05$). Values represent means ± standard error (n = 6).

3.6. *Ex vitro* morphological performance, growth, and biomass of plants derived from aCO_2 and eCO_2

After 50 days of *ex vitro* acclimatization, plants derived from *in vitro* culture under eCO_2 showed higher shoot and root development than plants derived from *in vitro* culture under aCO_2 (Fig. 8), as confirmed by quantitative growth parameters (Fig. 9).

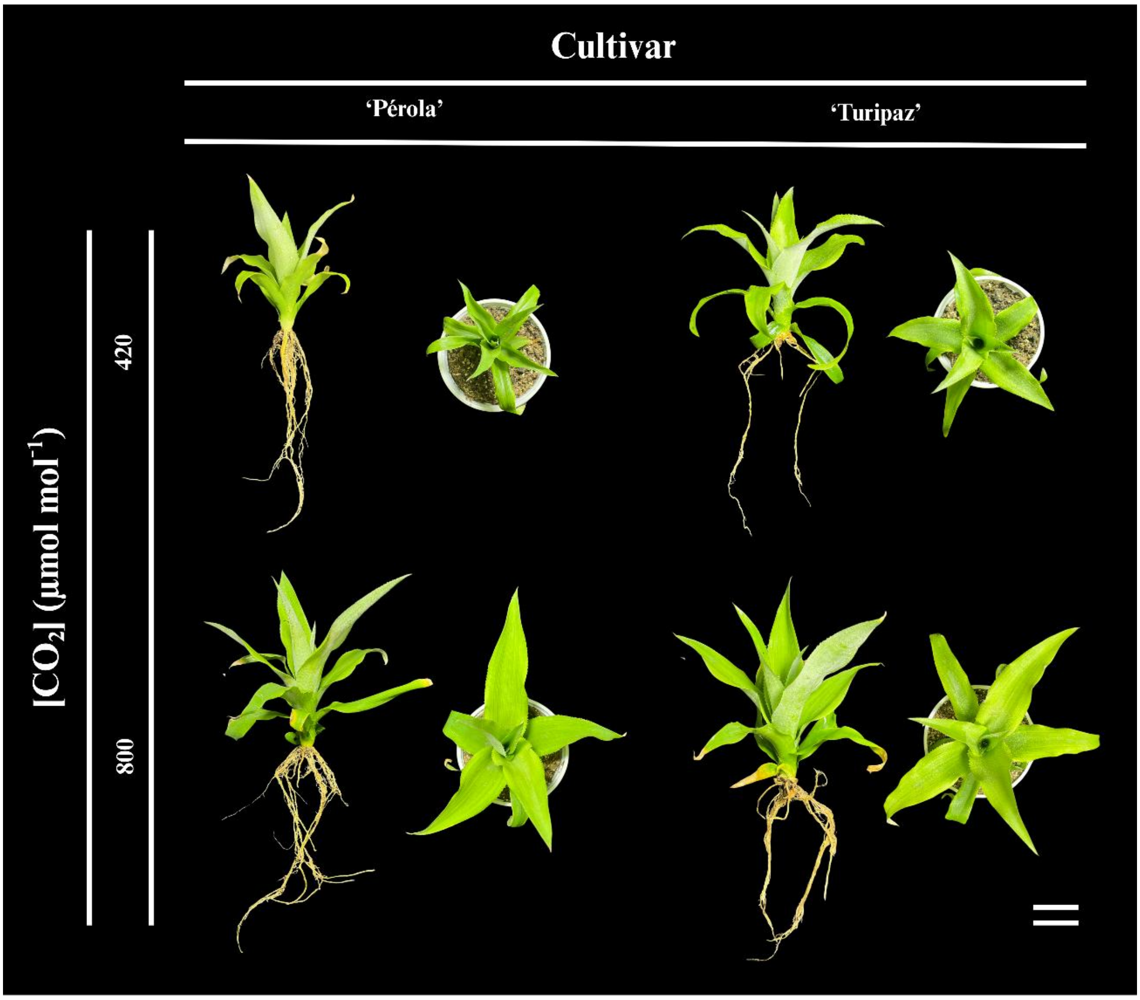

Fig 8. Morphological response of *Ananas comosus* plants of the cultivars 'Pérola' and 'Turipaz' following *in vitro* culture under two [CO_2] levels (420 and 800 ± 30 μmol mol^{-1}) and subsequent acclimatization for 50 days under *ex vitro* greenhouse conditions. Scale bar = 2cm

After *ex vitro* acclimatization, shoot length was higher in plants derived from *in vitro* culture under eCO_2, with greater values observed in the cultivar 'Turipaz' than in 'Pérola' (Fig. 9A). Root length also showed higher values under eCO_2, and 'Turipaz' exhibited slightly higher values than 'Pérola' across both CO_2 concentrations (Fig. 9B). The number of leaves was higher under eCO_2 in both cultivars, with higher leaf numbers observed in 'Turipaz' (Fig. 9C).

Rosette diameter showed higher values under eCO_2, with the highest values observed in 'Turipaz' (Fig. 9D). Fresh shoot biomass, fresh root biomass, and shoot dry biomass were higher under eCO_2, with consistently greater values in 'Turipaz' than in 'Pérola' (Fig. 9E–G). Root dry biomass did not differ between CO_2 concentrations; however, higher mean values were observed in 'Turipaz' during the *ex vitro* phase (Fig. 9H).

Leaf area was higher under eCO_2, with greater values observed in 'Turipaz' (Fig. 9I). Specific leaf mass also showed higher values under eCO_2, with consistently greater values in 'Turipaz' (Fig. 9J). In contrast, stomatal index showed lower values under eCO_2, while 'Turipaz' maintained higher mean values than 'Pérola' across CO_2 treatments (Fig. 9K).

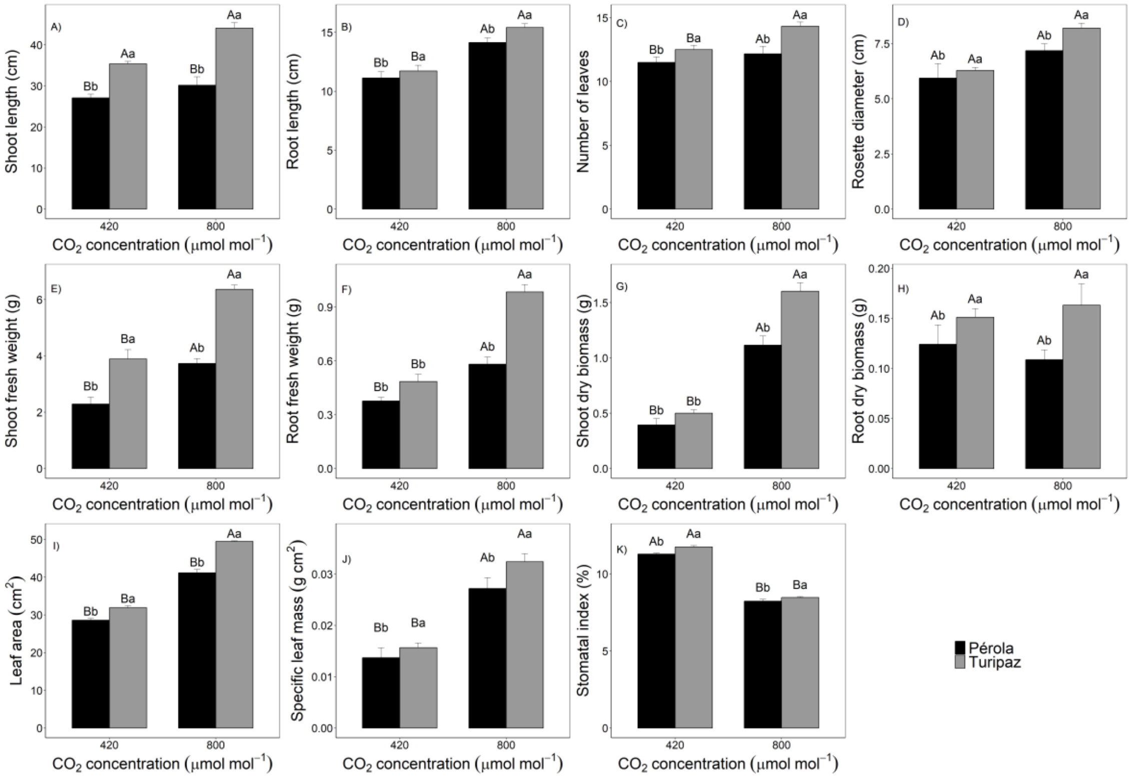

Fig 9. Growth, biomass, and stomatal index of *Ananas comosus* plants after 50 days of ex vitro culture. (A) Shoot length (cm); (B) root length (cm); (C) number of leaves; (D) rosette diameter (cm); (E) shoot fresh weight (g); (F) root fresh weight (g); (G) sho ot dry biomass (g); (H) root dry biomass (g); (I) leaf area (cm^2); (J) specific leaf mass (g cm^{-2}); and (K) stomatal index (%). Uppercase letters indicate significant differences between [CO$_2$] levels within the same cultivar, whereas lowercase letters indicate significant differences between cultivars within the same [CO$_2$] level (Tukey test, $P \leq 0.05$). Values represent means ± standard error ($n = 6$).

3.7.Ex vitro photosynthetic pigment dynamics of plants derived from aCO$_2$ and eCO$_2$

Under *ex vitro* conditions, chlorophyll a content was higher in plants derived from *in vitro* culture under eCO$_2$, with greater values observed in the cultivar ‘Turipaz’ than in ‘Pérola’ (Fig. 10A). Carotenoid content also showed higher values under eCO$_2$, with consistently greater values in ‘Turipaz’ compared with ‘Pérola’ (Fig. 10E).

Chlorophyll b content was higher under eCO$_2$ in both cultivars; under these conditions, ‘Pérola’ exhibited higher values than ‘Turipaz’ (Fig. 10B).

The chlorophyll a/b ratio showed lower values under eCO$_2$ during the *ex vitro* phase. Under aCO$_2$, significant differences between cultivars were observed, with higher values in ‘Turipaz’ (Fig. 10C).

Total chlorophyll content was higher under eCO$_2$, with greater values observed in ‘Turipaz’. In contrast, plants derived from aCO$_2$ showed lower total chlorophyll values during *ex vitro* growth (Fig. 10D).

The total chlorophyll-to-carotenoids ratio was higher under aCO_2, and during the *ex vitro* phase, higher values were observed in ‘Pérola’ compared with ‘Turipaz’ (Fig. 10F).

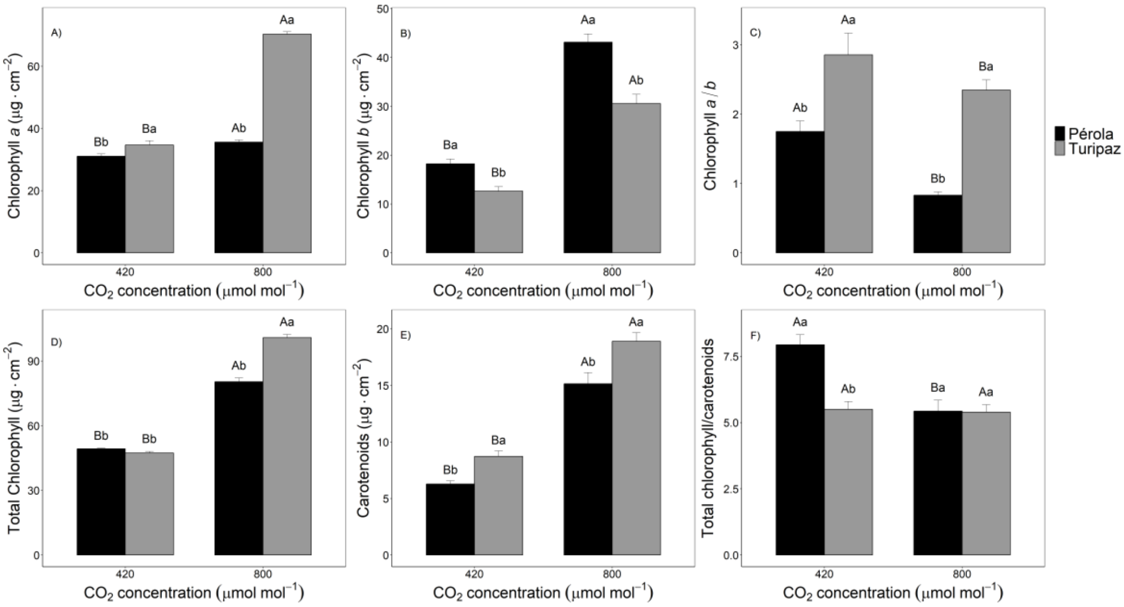

Fig 10. Photosynthetic pigments of *Ananas comosus* plants after 50 days of ex vitro culture. (A) Chlorophyll a (μg cm^{-2}); (B) chlorophyll b (μg cm^{-2}); (C) chlorophyll a/b ratio; (D) total chlorophyll (μg cm^{-2}); (E) carotenoids (μg cm^{-2}); and (F) total chlorophy ll/carotenoids ratio. Uppercase letters indicate significant differences between [CO_2] levels within the same cultivar, whereas lowercase letters indicate significant differences between cultivars within the same [CO_2] level (Tukey test, $P \leq 0.05$). Values represent means ± standard error (n = 6).

3.8.Ex vitro chlorophyll fluorescence of plants derived from aCO_2 and eCO_2

Under *ex vitro* conditions, Fv/Fm and RC/ABS did not differ between plants derived from *in vitro* culture under aCO_2 and eCO_2, with similar values observed across CO_2 treatments and cultivars (Fig. 11A, B). The performance index (PI) also did not differ between CO_2 concentrations during the *ex vitro* phase, however, differences between cultivars were detected, with higher PI values observed in ‘Turipaz’ compared with ‘Pérola’ across both CO_2 levels (Fig. 11C).

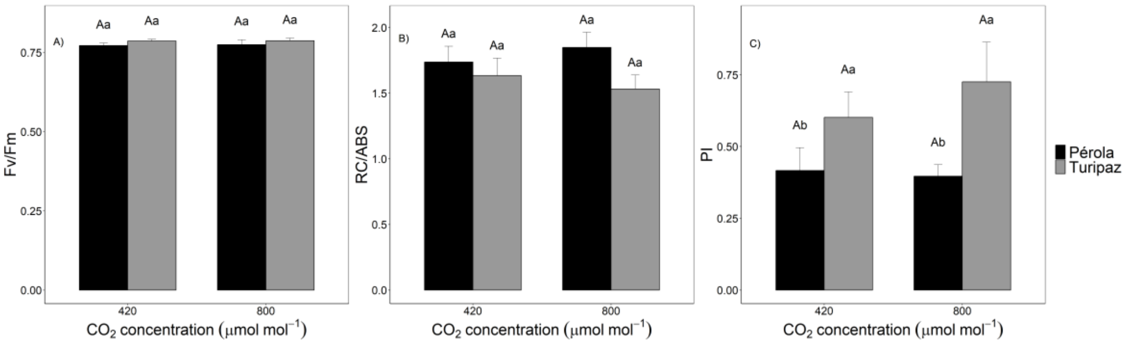

Fig 11. Chlorophyll fluorescence parameters of *Ananas comosus* plants after 50 days of *ex vitro* culture. (A) Fv/Fm; (B) RC/ABS; (C) performance index (PI). Uppercase letters indicate significant differences between [CO$_2$] levels within the same cultivar, whereas lowercase letters indicate significant differences between cultivars within the same [CO$_2$] level (Tukey test, $P \leq 0.05$). Values represent means ± standard error (n = 6).

3.9. Ex vitro soluble carbohydrate accumulation of plants derived from aCO$_2$ and eCO$_2$

Under *ex vitro* conditions, total soluble carbohydrate content showed higher values in plants derived from *in vitro* culture under eCO$_2$ than in those derived from aCO$_2$ (Fig. 12). No differences between cultivars were detected within each CO$_2$ level.

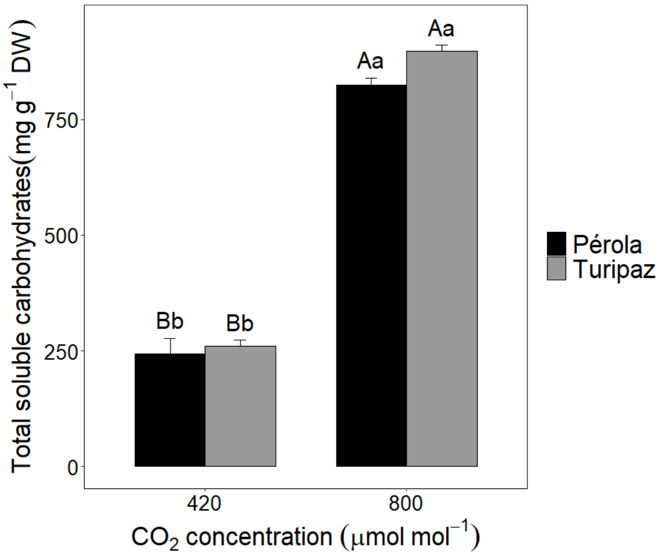

Fig 12. Total soluble carbohydrate content of *Ananas comosus* plants after 50 days of *ex vitro* culture. Uppercase letters indicate significant differences between [CO$_2$] levels within the same cultivar, whereas lowercase letters indicate significant diferences between cultivars within the same [CO$_2$] level (Tukey test, $P \leq 0.05$). Values represent means ± standard error (n = 6).

3.10. Ex vitro leaf micromorphometric traits of plants derived from aCO_2 and eCO_2

Under ambient aCO_2 conditions, both cultivars exhibited more compact mesophyll organization, with relatively smaller cells in both the chlorenchyma and aquiferous parenchyma. The tissues appeared more densely arranged, suggesting limited internal air space and reduced cell expansion. In contrast, under elevated eCO_2, a marked increase in cell size was observed, particularly in the aquiferous parenchyma, resulting in a more expanded and less compact mesophyll structure (Fig. 13A).

Comparatively, the ‘Turipaz’ variety showed more pronounced anatomical changes under elevated CO_2, with greater cell enlargement and a more heterogeneous tissue organization. The chlorenchyma appeared less compact and more loosely arranged, suggesting potential adjustments related to improved gas diffusion and photosynthetic activity. In ‘Pérola’, although cell expansion was also observed under elevated CO_2, the overall tissue organization remained relatively more uniform when compared to ‘Turipaz’ (Fig. 13A).

Under *ex vitro* conditions, abaxial epidermis thickness differed between plants derived from *in vitro* culture under aCO_2 and eCO_2 and between cultivars. Under aCO_2, higher values were observed in the cultivar ‘Turipaz’ compared with ‘Pérola’. Under eCO_2, no differences between cultivars were detected, with similar mean values observed. In ‘Turipaz’, abaxial epidermis thickness showed lower values under eCO_2 than under aCO_2 (Fig. 13B).

Adaxial epidermis thickness did not differ between CO_2 concentrations or cultivars, with similar values observed across treatments (Fig. 13C).

Aquifer parenchyma thickness showed higher values under eCO_2 in both cultivars, whereas no differences between cultivars were detected within each CO_2 level (Fig. 13D).

Chlorophyll parenchyma thickness differed between cultivars under aCO_2, with higher values observed in ‘Turipaz’. Under eCO_2, both cultivars exhibited higher and similar thickness values (Fig. 13E).

Transverse vascular bundle thickness showed higher values under eCO_2 in both cultivars, without differences between genotypes within each CO_2 concentration (Fig. 13F).

Longitudinal vascular bundle thickness differed between CO_2 concentrations and cultivars. Under aCO_2, higher values were observed in ‘Turipaz’ than in ‘Pérola’. Under eCO_2, both cultivars showed higher values, with ‘Turipaz’ maintaining higher mean values (Fig. 13G).

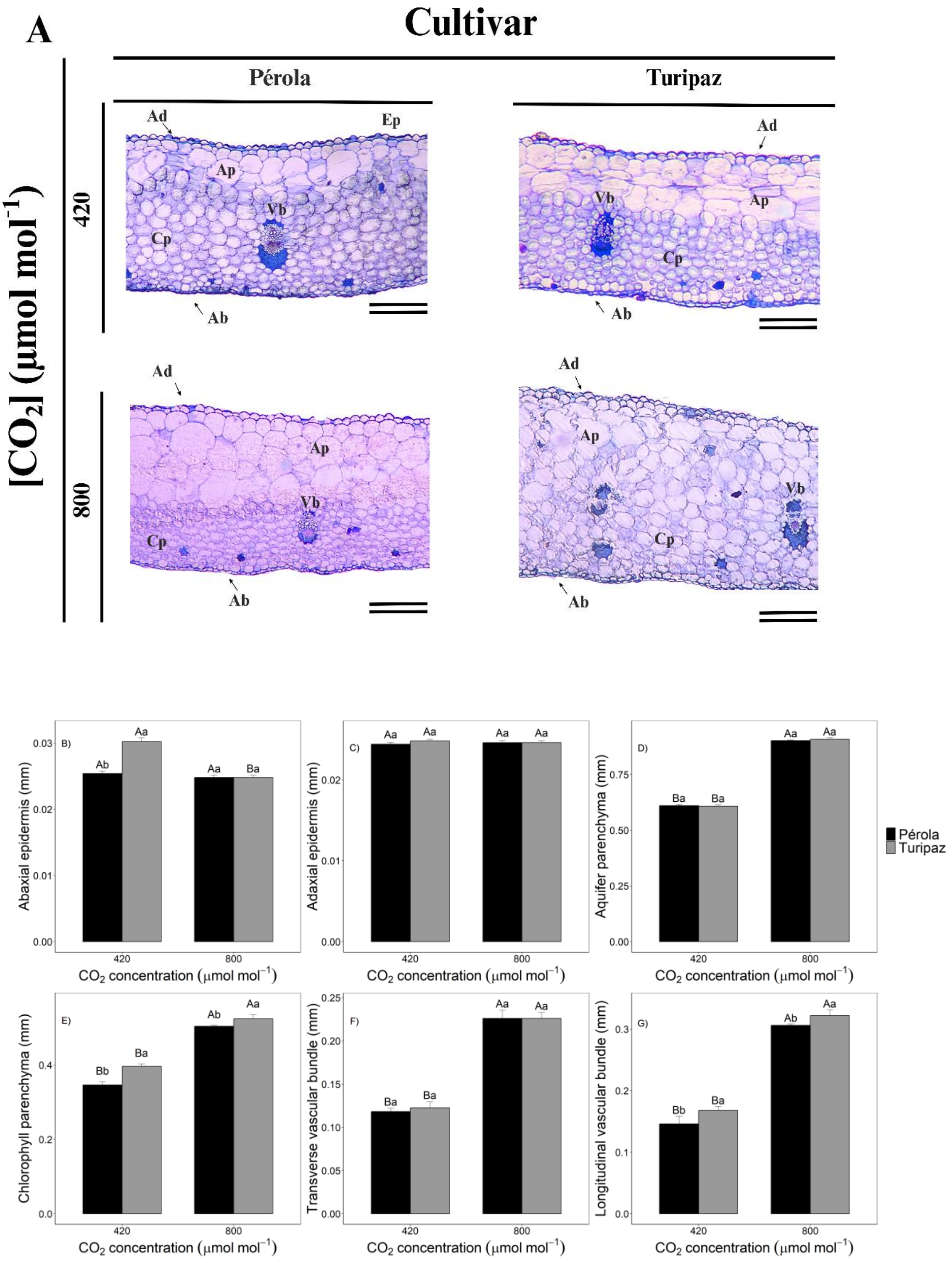

Fig 13. Leaf micromorphometric parameters of *Ananas comosus* plants after 50 days of *ex vitro* culture. (A) Leaf cross section (scale bar = 200µm); (B) Abaxial epidermis thickness (mm); (C) adaxial epidermis thickness (mm); (D) aquifer parenchyma thickness (mm); (E) chlorophyll parenchyma thickness (mm); (F) transverse vascular bundle thickness (mm); and (G) longitudinal vascular bundle thickness (mm). Uppercase letters indicate significant differences between [CO_2] levels within the same cultivar, whereas lowercase letters indicate significant differences between cultivars within the same [CO_2] level (Tukey test, $P \leq 0.05$). Values represent means ± standard error (n = 6).

4. Discussion

CO_2 enrichment in *in vitro* culture systems can promote shoot growth and bud emission, as evidenced here by the increased shoot length, particularly in the cultivar Turipaz (Fig. 3). Similar responses have been reported in *Vanilla planifolia*, in which elevated CO_2 concentration in temporary immersion systems promoted greater shoot elongation and leaf production, associated with increased activity of Rubisco and PEP carboxylase (Spinoso-Castillo and Bello-Bello, 2023). Comparable results were also observed in *Prunus domestica* cultivated in bioreactors under photomixotrophic and photoautotrophic conditions, where CO_2 enrichment resulted in enhanced vegetative growth and leaf area expansion (Gago et al., 2022). The contrasting responses between the cultivars Pérola and Turipaz reinforce the genotype-dependent nature of plant responses to elevated CO_2.

Root system development in both cultivars also responded positively to CO_2 enrichment, with increased root length and changes in relative growth patterns (Fig. 3). These findings corroborate evidence that adequate CO_2 availability in the *in vitro* environment is a key determinant of functional root development. In photoautotrophically grown *Cannabis sativa*, CO_2 enrichment enabled plantlet growth even in the absence of sucrose, resulting in more efficient root systems (Zarei et al., 2021). Additionally, increased gas exchange rates in culture vessels indirectly elevated internal CO_2 concentration, promoting biomass gains exceeding 140% and favoring shoot-to-root balance in *Cannabis sativa* (Shi et al., 2024).

Leaf expansion under eCO_2, expressed by higher leaf number, increased rosette diameter, and greater leaf área (Fig. 3), was consistent with responses reported in different species cultivated *in vitro* under CO_2 enrichment, including plum, torch ginger (*Etlingera elatior*), and coconut. In these species, increased CO_2 availability was associated with greater expansion of the photosynthetic surface and enhanced plantlet vigor (Gago et al., 2022; Pinheiro et al., 2021; Mu et al., 2025). These effects have been attributed to intensified net photosynthesis and optimization of carbon–energy balance, allowing greater investment in leaf tissues without compromising the growth of other organs.

The pronounced accumulation of fresh and dry biomass, particularly the more than twofold increase in shoot dry mass observed in both cultivars under eCO_2, indicates that CO_2 enrichment alleviates typical constraints of conventional *in vitro* culture (Fig. 3). Under low CO_2 availability, maximum quantum efficiency of photosystem II (F_v/F_m) is often compromised (Fig. 4), leading to reactive oxygen species accumulation and growth restriction even in the presence of sucrose (Askari et al., 2022). In contrast, elevated CO_2 concentration in a photoautotrophic system lacking exogenous carbon favors photosynthetic assimilation and efficient conversion of light energy into structural biomass (Trauger et al., 2022).

The reduction in stomatal index under eCO_2 was consistent with anatomical responses associated with plant growth under CO_2-enriched conditions (Fig. 3, Fig.7), where decreased stomatal density has been interpreted as an adaptive mechanism linked to improved water use efficiency without impairing carbon assimilation (Pinheiro et al., 2021). Studies with *Pfaffia glomerata* have similarly demonstrated that elevated CO_2 can induce relevant structural modifications, including changes in cell wall composition, resulting in more robust plants with improved acclimatization performance (Louback et al., 2021).

Leaf mass per area was not affected by CO_2 concentration in either cultivar (Fig. 3). This response partially contrasts with reports describing thicker leaves under elevated CO_2, such as in *Etlingera elatior* and *Ananas comosus* subjected to combined elevated CO_2 and salinity stress (Pinheiro et al., 2021; Silva-Moraes et al., 2025). These findings suggest that, in pineapple, CO_2 enrichment primarily favors leaf expansion and biomass accumulation without detectable changes in tissue density.

In the photoautotrophic *in vitro* system, CO_2 availability proved to be a determining factor for photosynthetic performance, acting directly to mitigate the diffusive limitation typical of culture vessels, as previously reported for pineapple (Alves et al., 2023; Silva et al., 2024). The increase in chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids under eCO_2 (Fig. 5) indicates that greater carbon availability supported the maintenance of the photosynthetic apparatus. The increase in chlorophyll b suggests expansion of light-harvesting complexes, while the stronger responses observed in Turipaz indicate greater physiological plasticity (Lim et al., 1998; Haisel et al., 1999; Alves et al., 2023; Silva et al., 2024).

Higher Fv/Fm values in Turipaz indicate greater integrity and potential efficiency of photosystem II, whereas the increase in RC/ABS suggests a higher density of active reaction centers. The performance index (PI) responded strongly to eCO_2 in both cultivars (Fig. 4), reflecting integrated improvement of photochemical efficiency and electron transport, consistent with previous reports (Wu and Lin, 2013; Alves et al., 2023; Pires et al., 2023). These photochemical improvements were mirrored by enhanced gas exchange, with increased net photosynthetic rate, stomatal conductance, internal CO_2 concentration, and Ci/Ca ratio under eCO_2, indicating intensification of diffusive CO_2 flux to the mesophyll (Lim et al., 1998; Pires et al., 2023; Silva et al., 2024). The higher transpiration observed particularly in Turipaz further supports genotype-dependent stomatal regulation.

The absence of significant changes in A/Ci and A/gs ratios indicates (Fig. 4) that CO_2 enrichment did not induce detectable alterations in biochemical carboxylation efficiency or intrinsic water use efficiency. Thus, increased photosynthetic assimilation can be primarily attributed to enhanced CO_2 availability and increased diffusive flux, in agreement with observations in CO_2-enriched *in vitro* systems (Saldanha et al., 2013; Silva-Moraes et al., 2025).

The substantial accumulation of total soluble carbohydrates under eCO_2 demonstrates that CO_2 enrichment favored carbon assimilation and storage in the photoautotrophic system (Fig. 6). In the absence of exogenous sucrose, CO_2 availability rapidly becomes limiting to photosynthesis, and increasing CO_2 concentration enhances photoassimilate production (Cournac et al., 1991; Kozai et al., 1997). In *Ananas comosus*, a CAM species, soluble sugars support nocturnal CO_2 fixation and organic acid dynamics (Carnal and Black, 1989), and their regulation is modulated by CO_2 availability (Borland et al., 2016; Chomthong et al., 2023). Although contrasting responses to elevated CO_2 have been reported for *A. comosus* (Zhu and Goldstein, 1999), the present results indicate that a photoautotrophic *in vitro* environment combined with elevated CO_2 enhanced carbon assimilation and soluble carbon accumulation.

Previous studies highlight that, in photoautotrophic systems, CO_2 availability and vessel gas exchange efficiency determine carbon assimilation, biomass accumulation, and physiological quality of plantlets, with direct consequences for ex vitro performance (Jeong et al., 1993; Kozai et al., 2005; Kirdmanee et al., 1995). In this context, the maintenance of growth gains after 50 days ex vitro, particularly in Turipaz, suggests greater autotrophic competence and increased availability of assimilates derived from eCO_2 culture.

Anatomical analyses further demonstrated that CO_2 enrichment during photoautotrophic *in vitro* culture was associated with consistent structural modifications in pineapple leaves (Fig. 7). Several of these responses persisted after acclimatization, indicating that conditions established during the *in vitro* phase influenced early tissue differentiation and were expressed in leaf anatomy under *ex vitro* conditions (Fig. 13). Similar patterns have been reported for pineapple and other species cultivated under elevated CO_2, in which *in vitro*-induced anatomical changes persist partially or fully after transfer to non-controlled environments (Pinheiro et al., 2021; Silva-Moraes et al., 2025).

During the *in vitro* phase, increased thickness of abaxial and adaxial epidermis under eCO_2 in both cultivars suggests that higher inorganic carbon availability was associated with epidermal structural modifications (Fig. 7). Under ambient CO_2 concentration, cultivar-dependent differences were observed, particularly for adaxial epidermis thickness, with higher values in Turipaz, whereas under eCO_2 these differences were not detected. This response pattern suggests that CO_2 enrichment can attenuate genotype-dependent anatomical differences under conditions of low inorganic carbon availability. Similar effects have been reported in *Ananas comosus* cultivated *in vitro* under elevated CO_2 combined with salinity stress (Silva-Moraes et al., 2025).

In the *ex vitro* phase, abaxial epidermis thickness varied with both genotype and *in vitro* CO_2 history (Fig. 13). Under aCO_2, Turipaz exhibited greater thickness than Pérola, whereas under eCO_2 values were similar between cultivars. In Turipaz, the reduced abaxial epidermis thickness in plants derived from eCO_2 compared with those from aCO_2 suggests that anatomical adjustments in this tissue may occur after acclimatization. In contrast, adaxial epidermis thickness did not differ among treatments or cultivars *ex vitro*, indicating lower plasticity of this tissue following transfer to external environmental conditions.

Aquifer parenchyma thickness showed a consistent response to CO_2 enrichment, with higher values under eCO_2 during both *in vitro* and *ex vitro* phases (Fig. 7, Fig. 13), without differences between cultivars. Given the CAM metabolism of pineapple, this response suggests that *in vitro* CO_2 conditions were associated with structural modifications in a tissue directly related to water storage and maintenance of cell turgor. The persistence of this pattern *ex vitro* indicates that aquifer parenchyma differentiation is sensitive to conditions established during *in vitro* culture, as also reported for *Etlingera elatior* (Pinheiro et al., 2021).

Chlorophyll parenchyma thickness responded to both genotype and CO_2 concentration. Under aCO_2, Turipaz exhibited greater thickness than Pérola during both phases, whereas under eCO_2 both cultivars showed similarly increased values, particularly *ex vitro* (Fig. 13). Comparable responses have been reported for *Vanilla planifolia* cultivated in CO_2-enriched *in vitro* systems, in which mesophyll development varied according to the gaseous environment during culture, reflecting differences in the organization of photosynthetic tissues (Spinoso-Castillo and Bello-Bello, 2023).

The vascular system also responded to CO_2 conditions applied during *in vitro* culture (Fig. 7). Increased thickness of transverse vascular bundles under eCO_2 in both phases and regardless of genotype indicates that the enriched environment was associated with structural modifications in conductive tissues. For longitudinal vascular bundles, responses were genotype dependent, with Turipaz exhibiting higher values than Pérola under eCO_2 during both phases. These findings indicate that, although CO_2 enrichment is associated with consistent anatomical changes, genotypic variability remains a determinant of vascular architecture, as also observed in micropropagated *Pfaffia glomerata* (Louback et al., 2021).

Collectively, these results demonstrate that CO_2 enrichment during photoautotrophic *in vitro* culture acts as a developmental regulator by preconditioning carbon balance, leaf structure, and physiological competence of pineapple plants (Fig. 7). Importantly, several of these responses persisted after transfer to *ex vitro* conditions (Fig. 13), highlighting the relevance of the *in vitro* gaseous microenvironment for shaping anatomical, physiological, and growth-related traits that contribute to improved acclimatization performance.

5. Conclusion

Our study demonstrates that CO_2 enrichment during photoautotrophic *in vitro* culture alleviates diffusive limitations inherent to closed culture systems, thereby enhancing growth, biomass accumulation, leaf expansion, and soluble carbohydrate content in *Ananas comosus*. These responses occurred without detectable changes in biochemical carboxylation efficiency, indicating that the observed gains were primarily driven by improved carbon availability and enhanced diffusive fluxes rather than by shifts in intrinsic photosynthetic efficiency.

The results further show that the *in vitro* gaseous microenvironment exerts a structuring influence on anatomical and functional traits, several of which persist during *ex vitro* acclimatization. By integrating growth, gas exchange, chlorophyll fluorescence, pigment composition, and carbon metabolism, this work fills a key knowledge gap by providing experimental evidence that CO_2 availability represents a controllable factor shaping carbon balance and physiological competence in photoautotrophic *in vitro* systems, with carryover effects extending beyond the *in vitro* phase and contributing to improved ex vitro establishment.

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