Journal of Plant Growth Regulation
https://doi.org/10.1007/s00344-026-12439-x

Effects of 24-Epibrassinolide Application on the Development of *Dipteryx odorata*: Between Growth Stimulation and Inhibitory Effects

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Received: 16 January 2026 / Accepted: 19 August 2026

Abstract

Dipteryx odorata, the Tonka Bean tree, is an Amazonian tree species of high ecological and economic importance, widely used in reforestation and restoration programs. This study evaluated the effects of exogenous application of 24-epibrassinolide (24-EBL) on the morphological and metabolic development of young seedlings of *D. odorata* cultivated under semi-controlled nursery conditions with 50% shading at the Federal University of Pará, Brazil. A total of 115 seedlings were distributed among five treatments corresponding to 0, 10, 20, 30, and 40 nM of 24-EBL. Morphological parameters (height, basal diameter, number of leaves, and height/diameter ratio) and leaf physicochemical parameters (chlorophyll, nitrogen, humidity, and leaf temperature) were monitored over five to six months. To explore treatment-related differences among individuals and choose the variables for statistical modeling, we performed a principal component analysis. The effects of the treatments on these variables were evaluated using linear mixed-effects models with maximum likelihood estimation. The responses were strongly dose-dependent. The 30 nM treatment promoted the best overall performance, significantly increasing height, basal diameter, leaf production, and chlorophyll content, while 40 nM produced the best height/diameter ratio, indicating improved seedling quality and structural stability. In contrast, the 20 nM treatment induced inhibitory effects characterized by reduced growth, decreased chlorophyll production, and signs of physiological stress, suggesting a possible sensitivity window associated with increased ethylene production. These findings indicate the potential of 24-EBL, particularly at 30–40 nM, as a promising biotechnological tool to improve seedling quality and optimize large-scale production of *D. odorata* for Amazonian reforestation and ecosystem restoration programs.

Keywords Brassinosteroids · Phytohormones · Seedling production · Amazonian species · Environmental restoration

Introduction

Dipteryx odorata (Aubl.) Forsyth F. (Fabaceae), known as Tonka bean tree or cumaru (Jaquetti and Gonçalves 2017), is a large tree species, reaching up to 30 m in height in primary forests, or less when cultivated in secondary forests (Silva et al. 2019). The species is present in approximately 30.41% of the entire territory of the state of Pará, the second largest federative unit of Brazil (Martins Santos et al. 2023). It occurs in Amazonian phytogeographic domains, including terra firme, seasonal semi-deciduous, and ombrophilous forests (Pinto et al. 2008), but also colonizes floodplains (Coradin et al. 2022). The species has an erect, cylindrical trunk, 50 to 70 cm in diameter, with thin, rough bark that peels off in irregular plates (Lorenzi 2002). The tree has heavy, high-quality wood with a density of 0.75 to 1.10 g/cm^3, and its timber is commonly called Brazilian Teak (Holm et al. 2014), used in the production of agricultural

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Published online: 05 September 2026

tools, shipbuilding, the manufacture of tool handles, posts, railroad ties, carts, stakes, supports, floors, beams, laminated woodwork items, and bearings for boat propeller shafts (Pinto et al. 2008; Silva et al. 2019). Its fruit is a dense, oval, woody legume with a late-dehiscent endocarp after decomposition of the mesocarp, 5–6.5 cm long and approximately 3.5 cm wide, with one or two seeds (Cruz and Barros 2021). Tonka beans are used to make necklaces and bracelets, in addition to being widely traded since the nineteenth century due to its excellent aroma, which is used to flavor chocolates, cigarettes, cigars, sweets, foods, and beverages, as well as in the production of perfumes, soaps, and other cosmetic products (Do Nascimento et al. 2022). The beans are aromatic due to the presence of coumarin, a bioactive molecule that influences various plant development processes, including germination, root growth, and stem elongation; in combination with brassinosteroids, coumarin is capable of inducing hypocotyl elongation, promoting plant growth (Van Praet et al. 2025). Cumaru (tonka) seed oil is extracted from the beans and widely used in perfumery, playing a significant role in Venezuela's regional economy and industry (Silva et al. 2019). For Brazil, the promotion of cumaru oil not only strengthens its position as a leader in the production and export of natural products, but also its identity on the international stage, contributing to sustainable development and environmental preservation (Bonfim et al. 2025).

Dipteryx odorata is a species with great potential for reforestation in the Eastern Amazon, as it offers important environmental, economic, and cultural resources (Do Nascimento et al. 2022). Cumaru cultivation is an economic alternative for family farmers in the western region of Pará, and the species is an effective alternative for forest restoration in degraded areas, reforestation, and soil recovery in abandoned pastures, as it was demonstrated in the municipality of Óbidos, in Eastern Amazon, Brazil (Mota et al. 2022). The species is among the priority species for conservation in the Amazon biome (Vieira et al. 2005), although it is vulnerable to negative impacts due to overexploitation, especially in the state of Pará (Cruz and Barros 2021).

Dipteryx odorata exhibits growth limitations in certain silvicultural systems. For example, in agroforestry systems combining cumaru with orange trees, the species displayed a comparatively low growth rate, with a slenderness ratio indicating unstable plantations requiring thinning practices (Godinho Da Silva et al. 2020). *Dipteryx odorata* also shows physiological sensitivity: when cultivated in nurseries, its growth may be influenced by shading, with or without associated water stress conditions (Uchida and Campos 2000). According to the International Union for Conservation of Nature (IUCN 2021), there is an urgent need to improve and expand ecosystem restoration, promoting scientifically proven practices and creating suitable conditions for restoration. In this context, the use of *D. odorata* is a promising strategy, as the species is recognized for its relevance in reforestation programs, productive capacity beginning at approximately four years of age (Pinto et al. 2008), and superior performance in growth and survival rate in four-year monospecific experimental plantations conducted under soil and climate conditions similar to those of the Brazilian Central Amazon (Machado et al. 2018). The use of phytohormones, such as brassinosteroids, is one of the most effective ways to reduce the production time of young plants to accelerate the reforestation process. Brassinosteroids regulate various processes in plant development and physiology, including seed germination, seedling photomorphogenesis, stomatal differentiation, organ boundary formation, flowering, male fertility, and responses to biotic and abiotic stresses (Wang et al. 2014). One of the advantages of using brassinosteroids is that they increase resistance to biotic and abiotic stresses, such as pathogens, extreme temperatures, saline soils, water scarcity, metal contamination, and nutritional deficiencies (Krishna et al. 2010; Bajguz et al. 2016).

Brassinosteroids are known to enhance production yields in several species essential for human consumption; they have been demonstrated to improve agricultural sustainability, productivity, yield, and growth performance in numerous economically important crop species (Sourabh et al. 2024). For example, they can increase seed productivity in common beans *Phaseolus vulgaris*, Kusha cultivar, COS16 genotype (Mohammadi et al. 2020). They can also play an important role in improving growth, biomass, productivity, yield, and reducing oxidative damage in wheat (*Triticum aestivum*) under drought conditions (Khan et al. 2021).

The existing literature on *D. odorata* does not address the impact of hormone use on the species. Therefore, our objective in this study was to evaluate the morphological and metabolic parameters of young *D. odorata* plants, verifying the effects of applying 24-epibrassinolide (24-EBL) at various concentrations (10, 20, 30, and 40 nM) on the growth and development of seedlings of the species under 50% shading. The central hypothesis of this experiment was that, in response to the application of 24-EBL, plants would exhibit a dose-dependent response, with lower concentrations (10–30 nM) optimizing morphological growth and promoting metabolic processes, while higher concentrations (40 nM) would not provide any additional benefits or even become less effective.

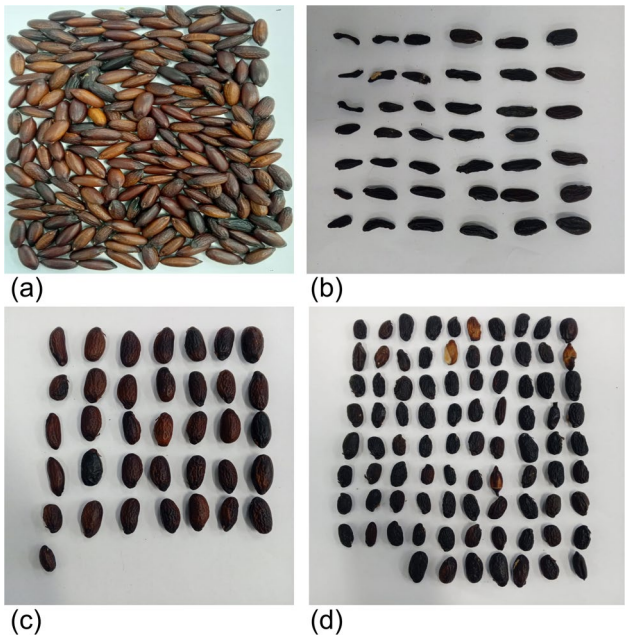

Fig. 1 Photos of different categories of *Dipteryx odorata* fruit seeds grouped prior to germination for the 24-epibrassinolide application experiment in the greenhouse at the Federal University of Pará, Altamira Campus. (**a**) Large, healthy fruit seeds; (**b**) seeds from insect-infested fruits; (**c**) light-colored seeds from healthy medium-sized fruit; (**d**) black seeds from healthy medium-sized fruit

Materials and Methods

Study Area

The experiment was conducted at the Federal University of Pará (Altamira, 3° 12′ 43″ S 52° 12′ 49″ W). According to the Köppen's climate classification, the region has a hot and humid tropical climate of types *Am* and *Aw*, with an average minimum annual temperature of 22.1 °C, an average maximum annual temperature of 32.4 °C, and annual precipitation of 2100 mm (Alvares et al. 2013). The plants were kept under 50% shade.

Seedling Production

The seedlings were produced from cumaru fruits supplied by the Institute for Forest and Biodiversity Development of the State of Pará (IDEFLOR-Bio), from which intact seeds were removed. The seeds, from four categories of fruits (Fig. 1), were placed in sand contained in tubes with a diameter of 4 × 15 cm; germination was monitored for 30 days. The characteristics and germination rates of each seed category differed greatly (Table 1).

A total of 140 plants were obtained from seeds in categories a and c; plants with inadequate shape and poor robustness were removed, and only 115 vigorous plants with the best morphological characteristics were selected for the experiment.

Soil Preparation

The 40-day-old seedlings were placed in black polyethylene plastic bags (15 × 25 cm and 20 µm in thickness), filled with a mixture of soil, fertilizer, organic matter, and Amafibra Golden Mix 47® commercial organic substrate. The mixture consisted of: 96 kg of coconut fiber, 12 kg of hillside soil, 1.5 kg of Yoorin® phosphate fertilizer, 1 kg of bone meal, 2 kg of NPK (4–14–8), 1.5 kg of Basacote® fertilizer (16–8-14), 3 kg of limestone, and 90 L of water. The technical report of the organic substrate's physical and chemical analysis can be found in the Supplementary File S1.

Experimental Design

The experiment was conducted in a randomized block design, with five replicates and five groups of 23 *D. odorata* plants aged 70 days, totaling 115 plants. The plants measured 7.0–19.9 cm in height, with stem diameters of 2.53–4.17 mm, and two to four leaves at the beginning of the experiment. The research was conducted between December 2024 and May 2025.

A single treatment application was performed. The control group (t0) did not receive the phytohormone application. The experimental treatments, named t1, t2, t3, and t4,

Table 1 Categories of *Dipteryx odorata* seeds cultivated according to their quantities, total weight, minimum and maximum weight, germination rate, and number of plants obtained for each category

Seed category	Quantity	Total weight (g)	Minimum weight (g)	Maximum weight (g)	Average weight (g)	Quantity of seedlings	Germination rate (%)
a	203	347.92	1.136	2.412	1.276	130	64
b	41	8.96	0.023	0.69	0.667	0	0
c	36	46.19	0.658	2.057	1.399	10	27.8
d	87	63.72	0.426	1.688	1.262	0	0
Total	367	466.79	-	-	-	140	-

received a single dose of 24-EBL, respectively, in four treatments consisting of doses of 10, 20, 30, and 40 nM of the hormone.

For the application of 24-EBL, we separated each group of plants with black plastic bags at a height of approximately 1.5 m to avoid spray drift. The application was performed with a constant pressure backpack sprayer, equipped with a lance and fan nozzle, manufactured by Kawashima Company, model PEM-P20. After application, the plants remained protected from rain for 24 h to prevent the wash-off of the applied hormone.

Measurements were performed at 30-day intervals, yielding six data points over a six-month period. Baseline data (M0) were collected prior to treatment, followed by five monthly assessments (M1–M5) after phytohormone application.

Data Collection

Starting in Month 0 (M0), plant height was measured using a 50-cm graduated ruler, the number of leaves was counted, and stem diameter was measured directly using a universal stainless-steel caliper (150 mm/6", accuracy ± 0.05 mm, Digimess®, Brazil). Starting in the second month (M1) and using a Goyojo® manual electronic chlorophyll meter, model GYJ-D, we measured leaf temperature, nitrogen, humidity, and chlorophyll indirectly using SPAD values. The values were collected in the morning between 7 a.m. and 8 a.m.

The collected data were classified into morphological variables (height, basal diameter, and number of leaves), representing plant development, and leaf physicochemical variables (SPAD chlorophyll, leaf nitrogen, leaf temperature, and leaf humidity), representing plant metabolism. Plant height in millimeters was divided by the average diameter (the sum of the basal diameters collected twice and on two different sides divided by two) to calculate the growth index, or height/diameter ratio, commonly used to assess seedling health (Haase 2008).

Statistical Analysis

We performed data analysis using the R v.4.5.1 language (R Core Team 2025) via RStudio v2025.05.1 Build 513 "Mariposa Orchid" (RStudio Team 2025). A first descriptive stage allowed us to characterize the distributions of the variables and assess normality, using descriptive statistics, and visualizing the distributions with histograms and quantile–quantile plots. In the case of non-normal distribution, the variables were transformed by natural logarithm to approximate normality before use in parametric models.

For analyses that allowed grouping by fixed and random factors in order to correct for pseudo-replication of data, the database used contained repeated measurements (n = 690) from the 115 individuals. For analyses requiring independent observations, an aggregate database per individual (n = 115) was created, where morphological and physicochemical leaf variables were calculated by subtracting the values collected at the beginning of the experiment from those collected at the end of the experiment (delta values, represented by Δ).

To explore the associations of differences in each individual by treatment and choose the variables for modeling, a principal component analysis (PCA) was conducted with the Δ values using the R base package, with post hoc tests performed with the *factoextra* (Kassambara and Mundt 2020) and *emmeans* (Lenth 2024) packages.

The PCA was used to select variables representative of hormonal treatment effects, after verifying that they met the requirements for use in parametric statistical models. Plant growth was represented by the height/diameter ratio, or growth index, after normalization by logarithmic transformation. SPAD chlorophyll, with normal distribution, represented photosynthetic capacity and nitrogen investment in the photosynthetic apparatus, indicating increasing potential for autotrophic carbon assimilation. Leaf temperature, with homogenous variance between groups verified with the Levene's test (Nordstokke et al. 2011), reflected the balance between absorbed radiation and cooling by transpiration, serving as an indicator of the water status of plants, their thermal load, and photosynthetic stress.

The effects of treatments on these variables were evaluated using linear mixed-effects models (LME) with restricted maximum likelihood estimation (REML) using the *lme4* package (Bates et al. 2015) on repeated measures, with time and treatments as fixed effects, and plant identity as a random effect to correct for pseudo-replication. The significance of the models was verified through analysis of variance (ANOVA), and pairwise mean comparisons were verified through Tukey's method (Tukey 1949), using *emmeans* and *lmertest* (Kuznetsova et al. 2017) packages. Akaike information criteria (AIC) and marginal and conditional R^2 values of the models were calculated using the *MuMIn* package (Barton 2025), and the explanatory power was calculated using the *performance* package (Lüdecke et al. 2021). The homoscedasticity of the adjusted values and residuals of the models were examined post hoc by Breusch-Pagan tests (Breusch and Pagan 1979). The *p*-values of hypothesis tests were adjusted by the Holm-Bonferroni method (Holm 1979).

Graphical representations were constructed using the *ggplot2* package (Wickham 2016), included in the *tidyverse*

package (Wickham et al. 2019). Multiple graphs were constructed using the *patchwork* package (Pedersen 2024).

Results

Overall Development of *Dipteryx odorata* Plants in Experimental Cultivation

All 115 *Dipteryx odorata* plants grown under experimental conditions survived throughout the study. The characteristics measured during the six-month study showed heterogeneous development when grouped by treatment (Fig. 2). There was no significant morphological variation in the treatments after the first 30 days. With regard to morphological characteristics such as height, number of leaves, and diameter (Fig. 2a-c), treatment t3 showed the best results from M2 until the end of the experiment. The plants were slightly taller on t3 than those under treatment t4; diameters were larger than the other treatments and, from M4 onwards, the number of leaves was larger than in the other treatments. Treatment t4 showed the best gains in height/diameter ratio, which is also affected by height (Fig. 2d).

Regarding phyto-physicochemical variables such as chlorophyll, leaf moisture, and nitrogen (Fig. 2e-g), treatment t3 showed better results until time M4, being then surpassed by t0 at time M5. Considering all variables, except leaf temperature, both morphological and phyto-physicochemical, treatment t2 showed marked limiting effects. From M2 (60 days after 24-EBL application), there was a marked effect of t2 on plant height, with high frequencies at the lowest values, indicating dwarfism or growth suppression. At M4, t4 plants were substantially larger than t0 and t1 plants, but this difference disappeared after one month, leaving only treatment t2 with inhibitory effects. The number of leaves also showed an inhibitory effect of t2, when compared to the other treatments.

Leaf temperature developed differently from other leaf measurements (Fig. 2h), presenting an asymmetric distribution, with all treatments exhibiting similar variation and a drop in measurements in months M3 and M4. This phenomenon does not appear to be related to the treatments, but rather to natural environmental conditions.

Principal Component Analysis (PCA)

The principal component analysis (PCA), corrected for repeated measurements of individuals, revealed clear multivariate patterns in the response of plants to different treatments. The first two components together explained 68.2% of the total variability (PC1 = 40.0%; PC2 = 28.2%) and indicated a clear separation between the experimental groups (Fig. 3a). PC1 was strongly associated with the height, diameter, and number of leaves of individual plants, while PC2 mainly reflected variations related to leaf nitrogen and chlorophyll (Table 2, Fig. 3b). Height, diameter, and growth were positively associated with leaf temperature, while leaf nitrogen, chlorophyll, and humidity were inversely related to this axis. Multivariate analysis of variance (MANOVA) indicated significant differences between treatments (MANOVA: Pillai = 0.582; $F_{(8,220)} = 11.30$; $p < 0.001$), and both PC1 and PC2 showed significant treatment effects ($p < 0.001$ and $p = 0.001$, respectively; Supplementary Table S1). Contrasts between groups were confirmed by Tukey's multiple comparison test (Supplementary Table S2).

There were three distinct groups of variables that behaved cohesively in the PCA: morphological variables (height, diameter, number of leaves, and height/diameter ratio, or growth index) had similar directions and high contributions to explaining variability between groups, totaling 53.73%. Three of the leaf biochemical characteristics (SPAD chlorophyll, nitrogen, and humidity) varied concomitantly, with very close loading values and a total contribution of 45.63%. Leaf temperature, on the other hand, with a low contribution to explaining the variability between groups (0.65%), showed an opposite direction to the other variables. Thus, to reduce dimensionality in hypothesis testing, the response variables chosen for the models – representing the three groups of variables – were growth index (height/diameter ratio), leaf chlorophyll, and leaf temperature. Chlorophyll showed normal distribution, the growth index approached normality after natural logarithm transformation, and leaf temperature showed homoscedasticity within treatments, allowing these three response variables to be used in parametric models.

Effect of 24-EBL Treatments on the Growth of *Dipteryx odorata* Plants

A linear mixed-effects model (LME/REML) revealed significant effects of treatments ($F_{4,110} = 6.41$; $p = 0.001$), time ($F_{5,550} = 129.27$; $p < 0.0001$), and their interaction ($F_{20,550} = 5.22$; $p < 0.0001$) on the height/diameter ratio, with effects controlled for each individual. The model explained a moderate proportion of the variance through fixed effects (marginal $R^2 = 35.8\%$) and a high proportion when random effects were included (conditional $R^2 = 76.1\%$). The model fit indices indicated good performance (AIC = −428.57; BIC = −284.82; log-likelihood = 246.29; Table 3). The complete values of random and fixed effects can be seen in Supplementary Table S3. The model residuals achieved homoscedasticity (Breusch-Pagan: BP = 2.72; $p = 0.099$),

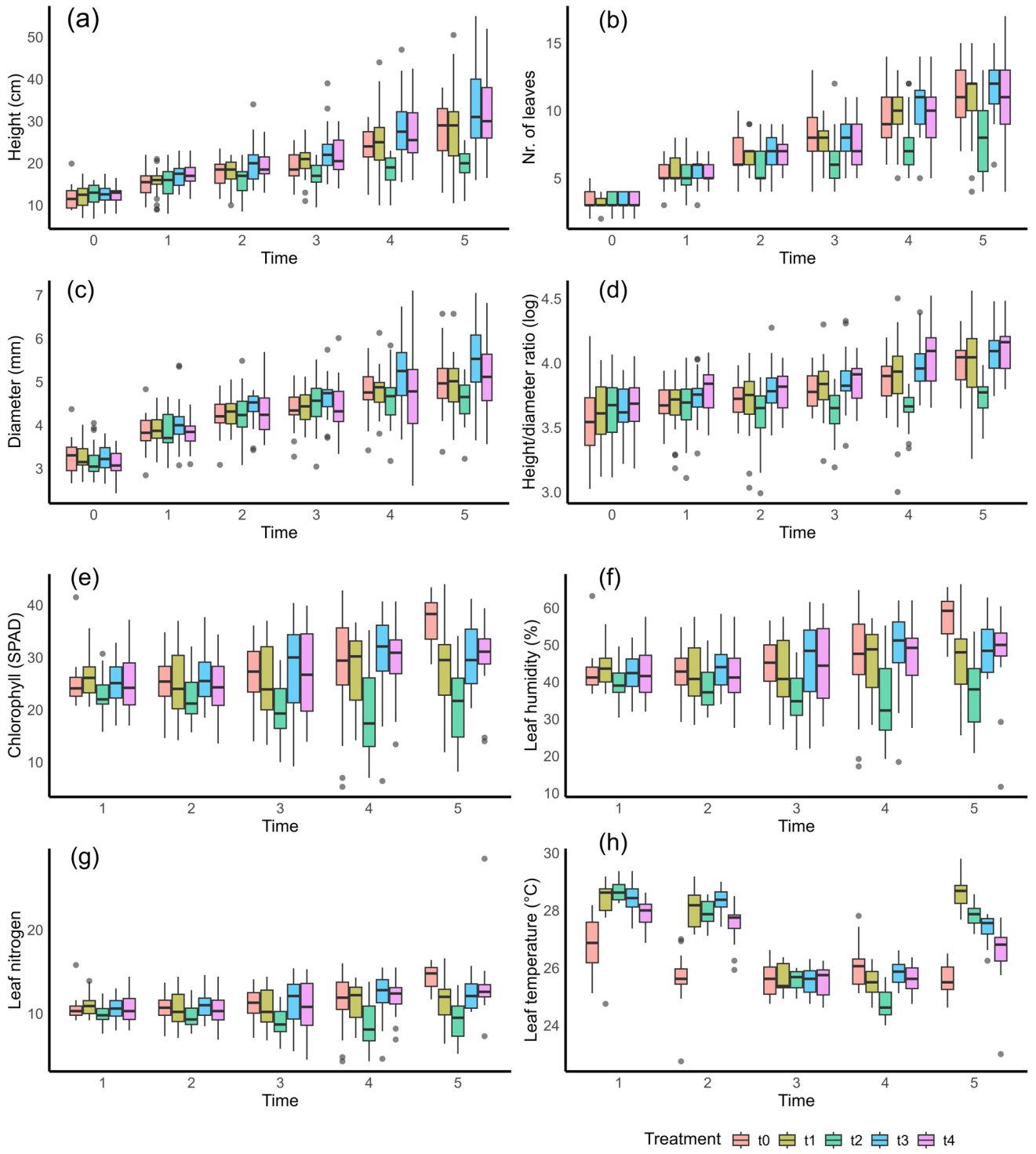

Fig. 2 Distribution of morphological and phyto-physicochemical variables, separated by treatment and time, from 115 *Dipteryx odorata* plants grown experimentally in the UFPA-Altamira nursery under 50% shade and different concentrations of the phytohormone 24-epibrassinolide for six months. (**a**) plant height in cm; (**b**) number of leaves; (**c**) average basal diameter in mm; (**d**) height/diameter ratio; (**e**) SPAD chlorophyll index; (**f**) leaf humidity in %; (**g**) leaf nitrogen in mg/g; (**h**) leaf temperature in °C. Horizontal bars represent means, vertical bars (whiskers) represent data range and gray dots represent outliers

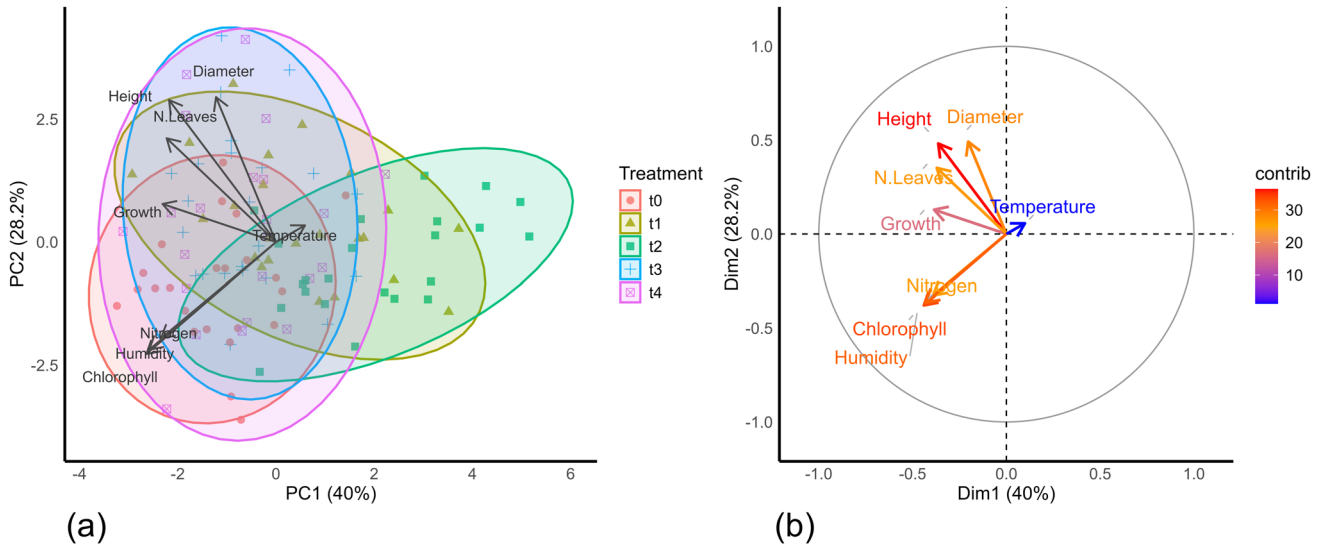

Fig. 3 Principal component analysis (PCA) of the relationships between morphological and biochemical variables from data collected from *Dipteryx odorata* plants grown experimentally under different concentrations of the phytohormone 24-epibrassinolide for six months. (**a**) Biplot of the distribution of individuals by data and treatment, organized in the first two components of the analysis; (**b**) loadings and contributions of the variables in the first two components of the analysis

Table 2 Variables used in principal component analysis (PCA), loadings of the two main components, and contributions, in percentage, of each variable, from data collected from *Dipteryx odorata* plants grown experimentally under different concentrations of the phytohormone 24-epibrassinolide for six months

Variable	PC1	PC2	Contribution (%)
Height (cm)	-0.3631	0.4819	18.21
Diameter (mm)	-0.2036	0.4918	14.17
Height/diameter ratio	-0.3853	0.1293	8.26
Number of leaves	-0.3712	0.3522	13.09
Chlorophyll (SPAD)	-0.4399	-0.3788	16.85
Nitrogen (mg/g)	-0.3902	-0.3256	12.91
Temperature (°C)	0.0986	0.05667	0.65
Leaf humidity (%)	-0.4301	-0.3639	15.87

and the graph of adjusted values against residuals did not show clear patterns (Supplementary Figure S1).

Post hoc paired comparisons were performed using estimated marginal means and Tukey's adjusted paired comparisons, and revealed no significant differences between treatments in the first two months of the experiment (M0 and M1; all $p > 0.40$). Differences between treatments began to emerge from the third month (M3), when plants under treatment t2 became significantly smaller than those under other treatments, and these differences increased in magnitude over the following months. In the last two months (M4 and M5), treatment t2 consistently showed significantly lower estimated marginal means than all other treatments ($p < 0.05$), while no significant differences were detected in the height/diameter ratio between treatments t0, t1, t3, and

Table 3 Summary and adjustment measures of the LME/REML model of the effects of the experimental application of four concentrations of 24-EBL on the height/diameter ratio of 115 *Dipteryx odorata* plants monitored for six months

LME/REML Model: Height/diameter ratio ~treatment*Time (1|ID)				
Marginal R^2			0.358	
Conditional R^2			0.761	
AIC			-428.57	
BIC			-284.82	
LogLik			246.29	
Model ANOVA				
Term	NumDf	DenDf	F	*p*
(Intercept)	1	550	55,267.50	<0.0001****
Treatment	4	110	6.41	0.001***
Time	5	550	129.27	<0.0001****
Treatment:Time	20	550	5.22	<0.0001****

NumDf = degrees of freedom of the numerator; DenDf = degrees of freedom of the denominator

Significance levels of p values * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001.

t4. These findings confirm that the divergence in treatment level depended on time and only became statistically distinguishable after the first measurement periods. The estimated marginal means, with standard errors and lower and upper confidence intervals (95% CI), can be seen in Supplementary Table S4 (Supplementary Figure S2). The difference between the first and last measurements (Δ) in each experimental group can be seen in Fig. 4a. Paired contrasts over time (adjusted by Tukey) with significance levels can be seen in Supplementary Table S5 and are summarized in Fig. 4b.

Effect of 24-EBL Treatments on the Phyto-Physicochemical Characteristics of *Dipteryx odorata* Plants

Two leaf physicochemical characteristics were tested with LME/REML: SPAD chlorophyll, which showed normal distribution, and leaf temperature, after verification of homoscedasticity (Levene's test: $F_{4,110} = 0.8351$; $p = 0.5057$) for experimental groups.

For SPAD chlorophyll, the linear mixed effects model was highly significant (constant: $F_{1,550} = 5150$; $p < 0.0001$), also with strong treatment effects ($F_{4,440} = 14.641$; $p < 0.0001$), time ($F_{4,440} = 14.947$; $p < 0.0001$), and the interaction between treatment and time ($F_{16,440} = 4.197$; $p < 0.0001$). As with the effects on plant growth, fixed effects moderately explained the variance (marginal $R^2 = 26.8\%$), and the explanatory power increased with the inclusion of random effects (conditional $R^2 = 43.7\%$), despite the lower values. However, the model had less power than the previous one, with higher adjustment values (AIC = 3676.871; BIC = 3793.238, log-likelihood = −1811.435; Table 4). The complete values of the random and fixed effects can be seen in Supplementary Table S6. Residuals showed homoscedasticity (Breusch-Pagan: BP = 0.229; $p = 0.6317$) and did not show visible patterns against adjusted values (Supplementary Figure S3).

The application of 24-EBL influenced the physiological activity of treated plants during the period. Post hoc paired comparisons were performed using estimated marginal means and Tukey's adjusted paired comparisons, and revealed no significant differences between treatments in the first two months of the experiment (M1 and M2; all $p > 0.40$). The significant difference between t2 and the other treatments appears from M3 until the end of the experiment (Supplementary Table S8).

From M1 to M5, there was no variation in chlorophyll production between t3 and t4. From M1 to M4, there was no significant difference in chlorophyll production between t0 and treatments t1, t3, or t4. Chlorophyll increase reached a maximum of 44 in group t0 in the control treatment, which completely separated the distribution of the group's data. Chlorophyll production in t1 remained below t0 from M1

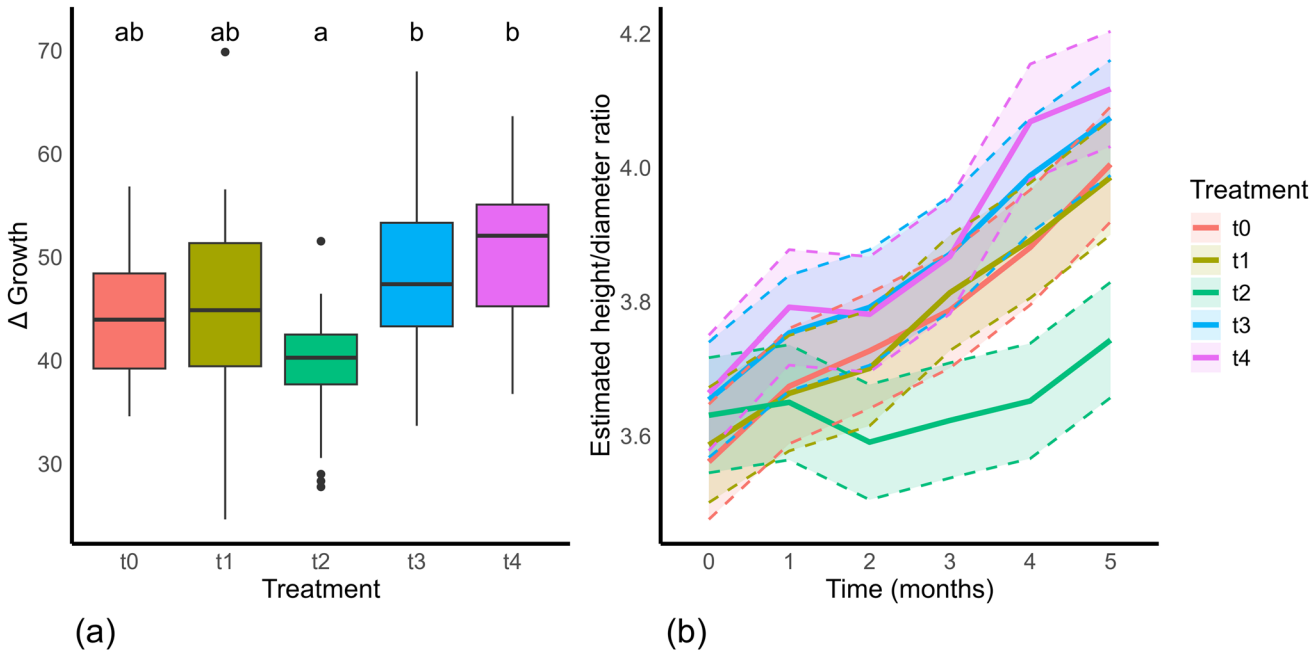

Fig. 4 (**a**) Boxplot of the difference between growth rates of *Dipteryx odorata* plants subjected to different concentrations of 24-EBL (t0 = control group) over six months of experimentation. Horizontal bars represent means; dots represent outliers. Letters result from paired comparisons adjusted by Tukey's test (α = 0.05). Treatments sharing letters belong to the same statistical group; the absence of common letters indicates significant differences. (**b**) Tukey-adjusted estimated measures of marginal means of treatments across months. Areas between dotted lines represent upper and lower limits of the 95% confidence interval

Table 4 Summary and model fit measures for the LME/REML model of the effects of the experimental application of four concentrations of 24-EBL on the leaf SPAD chlorophyll levels of 115 *Dipteryx odorata* plants monitored for five months

LME/REML Model: Chlorophyll ~treatment*Time (1|ID)				
Marginal R^2		0.268		
Conditional R^2		0.437		
AIC		3676.871		
BIC		3793.238		
LogLik		-1811.435		
Model ANOVA				
Term	NumDf	DenDf	F	*p*
(Intercept)	1	440	5159.072	<0.0001****
Treatment	4	110	14.641	<0.0001****
Time	5	440	14.947	<0.0001****
Treatment:Time	16	440	4.197	<0.0001****

NumDf = degrees of freedom in the numerator; DenDf = degrees of freedom in the denominator

Significance levels of *p* values * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001.

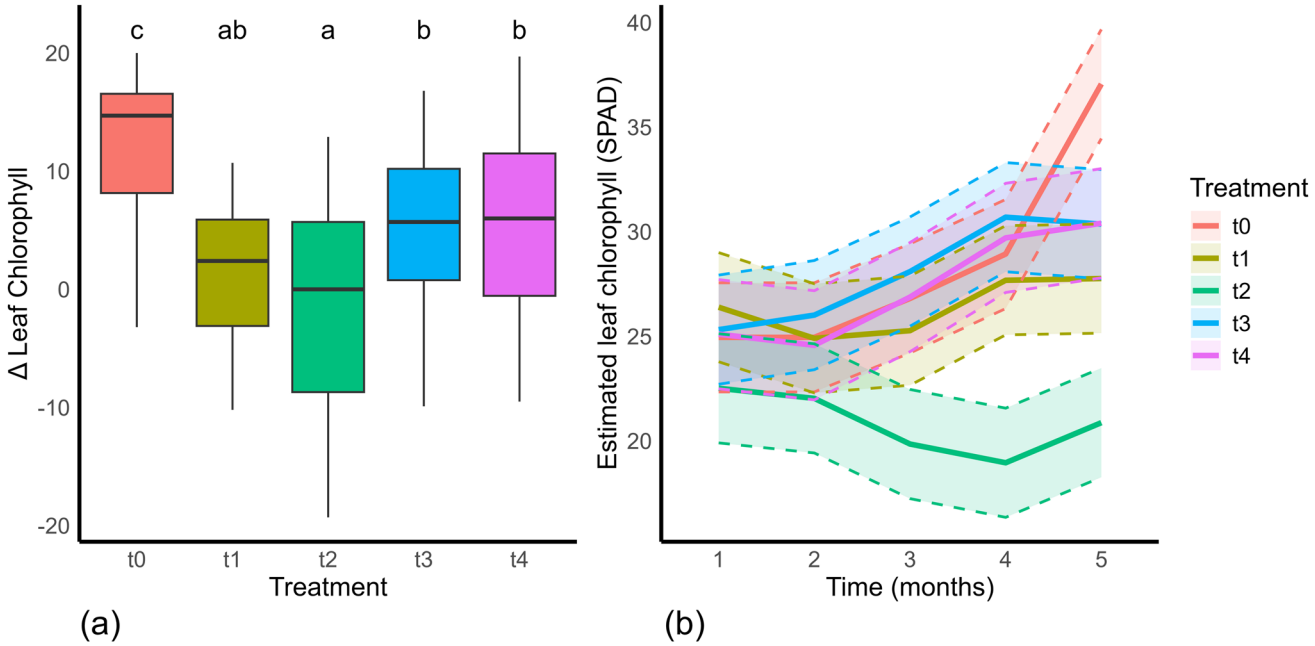

Fig. 5 (**a**) Boxplot of the difference between leaf SPAD chlorophyll levels of *Dipteryx odorata* plants subjected to different concentrations of 24-EBL (t0 = control group) over five months of experimentation. Horizontal bars represent means; dots represent outliers. Letters result from paired comparisons adjusted by Tukey's test (α = 0.05). Treatments sharing letters belong to the same statistical group; the absence of common letters indicates significant differences. (**b**) Tukey-adjusted estimated measures of marginal means of treatments across months. Areas between dotted lines represent upper and lower limits of the 95% confidence interval

to M3, and t2 (having the lowest chlorophyll production) with an increasing inhibitory effect from M1 to M4. The contrast between t0 and all other treatments is significant (Supplementary Figure S4). The estimated marginal means, with standard errors and lower and upper confidence intervals (95% CI), can be seen in Supplementary Table S7 (Supplementary Figure S4). The control group (t0) showed the greatest differences in chlorophyll between the beginning and end of the experiment (Δ), while groups t1 and t2 showed the smallest differences (Fig. 5a). Over time, group t3 had higher levels of leaf chlorophyll, with a drop after the fourth month (M3) in its estimated values. More visible was the inhibitory effect of treatment t2, with much lower levels of chlorophyll estimated over time (Fig. 5b). Paired contrasts over time (adjusted by Tukey) with significance levels can be seen in Supplementary Table S8.

Regarding leaf temperature, which showed peculiar development in months M3 and M4, weather seemed to be a much more influential factor than the effect of treatments on the measured levels (Table 5, Supplementary Table S9), although plants in treatment t1 showed a clear difference in leaf temperature at the end of the experiment (Fig. 6a). The

Table 5 Summary and adjustment measures of the LME/REML model of the effects of the experimental application of four concentrations of 24-EBL on leaf temperature (in °C) of 115 *Dipteryx odorata* plants monitored for five months

LME/REML Model: Leaf temperature ~treatment*Time (1|ID)				
Marginal R^2		0.825		
Conditional R^2		0.831		
AIC		1085.115		
BIC		1201.482		
LogLik		-515.5573		
Model ANOVA				
Term	NumDf	DenDf	F	*p*
(Intercept)	1	440	1,076,642.6	<0,0001****
Treatment	4	110	78.8	<0,0001****
Time	5	440	471.8	<0,0001****
Treatment:Time	16	40	34.3	<0,0001****

NumDf = degrees of freedom of the numerator; DenDf = degrees of freedom of the denominator

Significance levels of *p* values * ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001; **** ≤ 0.0001.

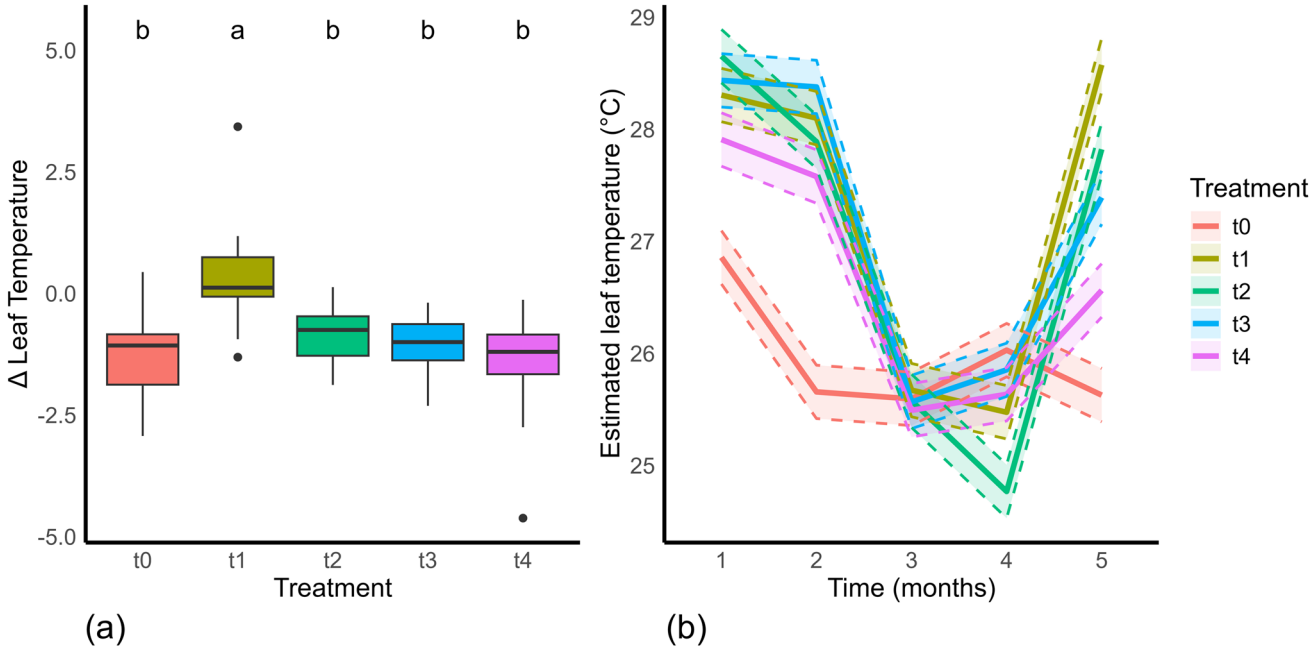

Fig. 6 (**a**) Boxplot of the difference between leaf temperature (in °C) of *Dipteryx odorata* plants subjected to different concentrations of 24-EBL (t0 = control group) over five months of experimentation. Horizontal bars represent means; dots represent outliers. Letters result from paired comparisons adjusted by Tukey's test (α = 0.05). Treatments sharing letters belong to the same statistical group; the absence of common letters indicates significant differences. (**b**) Tukey-adjusted estimated measures of marginal means of treatments across months. Areas between dotted lines represent upper and lower limits of the 95% confidence interval

LMER/REML model indicated that most of the variation was explained by fixed effects (marginal R^2 = 0.825), with minimal contribution from random effects (conditional R^2 = 0.832). The main effect of time was highly significant, reflecting sharp reductions in leaf temperature in months 3 and 4, followed by recovery in month 5, as shown by marginal means estimates (Supplementary Table S10; Fig. 6b). The Treatment × Time interaction was also significant, indicating that differences between treatments varied across months; in particular, no differences between treatments were detected in M3 (Figure S6), while in the other months there were significant contrasts (Supplementary Table S11).

Estimates of coefficients, standard errors, and *p*-values are detailed in Table S9. Paired contrasts adjusted by Tukey's method are presented in the Supplementary Table S11. Figure 6a shows the differences (Δ) in leaf temperature throughout the experiment between treatments. Verification of the assumptions indicated adequate homoscedasticity (Breusch–Pagan: BP = 30.061; p = 0.1827), although the residual plot shows a slight pattern associated with months (Supplementary Figure S5).

Regarding the decrease observed in leaf temperature, according to the Meteorological Database (BDMEP) of the Brazilian National Institute of Meteorology (INMET), rainfall in the region varied between 68.8 mm and 444.6 mm, while the mean environmental temperature varied between 20.9 °C and 32.5 °C. Precipitation in the region was 112.2 mm in M0, increasing to 444.6 mm in M1, with high values observed between M1 and M4, followed by a decrease to 68.8 mm in M5. As for the environmental temperature, the minimum values recorded were between M1 and M4. Monthly rain days in the city of Altamira from 1990 to 2019 averaged from the lowest of 17 days in December to 21 days in March. In the same historic period, the average hours of sunlight were the highest in December (7.4 h/day) and the lowest in April (4.8 h/day) due to cloud cover. The relevant data can be found in Supplementary Table S12.

Discussion

This study investigated the impact of different doses of 24-EBL on the growth and development of young *Dipteryx odorata* plants. Our results demonstrate that 24-epibrassinolide significantly influences the early development of *Dipteryx odorata*, with the strongest growth stimulation observed at 30 nM, whereas the 20 nM concentration produced inhibitory effects. Low germination rates were observed in seeds from insect-infested fruits and in dark seeds from medium-sized fruits, whereas seeds from large, healthy fruits are essential for the production of seedlings with greater vigor and better morphological characteristics. Perea et al. (2020) demonstrated that seed size and the differential localization of associated damage determine the occurrence of embryonic lesions and the specific region affected within the embryo (e.g., the plumule or radicle), which, in turn, influences key components of plant vigor. It was demonstrated that, in this species, the morphology of seedlings during germination, especially collar diameter, is a reliable indicator of the physiological quality of seeds, being strongly correlated with root biomass, total biomass, and the overall vigor of seedlings, directly reflecting the germination performance of the species (Guimarães et al. 2024). The germination of *Dipteryx odorata* seeds results from physiological processes involving imbibition, metabolic activation, and hormonal regulation, dominated by the balance between abscisic acid and gibberellins, which control dormancy breaking, reserve mobilization, and radicle elongation, leading to seedling emergence (Bargah et al. 2025).

In this experiment, a much more noticeable effect was observed with high concentrations of 30 nM for the best gain in size, plant diameter, leaf production, and chlorophyll production; and 40 nM for the best plant growth rate. Limiting effects on growth and chlorophyll production were observed for this species, with a sensitivity window around 20 nM of 24-EBL. This was manifested by plants with less developed stem diameter, shorter heights, narrower leaves and chlorosis.

Effects of Treatment on the Morphological Characteristics of *Dipteryx odorata* Plants

In this study, significant morphological responses were detected 60 days after hormone application, indicating that the response of D. odorata to 24-EBL may occur relatively slowly compared with other species. Kumar et al. (2024) and Yuan et al. (2017), respectively, observed similar results when evaluating the physio-morphological differences in responses related to the application of 24-EBL after 7 to 28 days in corn (*Zea mays* L.) and 15 days in foxtail millet (*Setaria italica* L.). The impact of the hormone would therefore depend on parameters such as dose, method of application (foliar, root, or injectable), and stage of plant development, which play an important role in the timing of morphological changes.

In *D. odorata*, increased size, leaf production, and diameter were observed in plants treated with 30 nM of the hormone. Yuan et al. (2017) showed that foliar application of 24-EBL can increase plant height. Plants treated with 40 nM showed the best growth rate. This concurs with the positive effect of 24-EBL on improving the morphological parameters of *D. odorata*. Similar studies conducted on *Ananas comosus* also showed that brassinosteroids increase the length, diameter, number of leaves, fresh and dry weight of the aerial parts of plants (Freitas 2010). These results highlight the importance of using 24-EBL to improve the biological parameters of young *D. odorata* plants for qualitative production with improved and beneficial growth yields, especially at optimal seasons to facilitate reforestation processes.

Plants treated with 20 nM of the hormone exhibited inhibitory effects characterized by slow growth in terms of diameter, size, and number of leaves. This highlights the existence of a sensitivity window around which 24-EBL would likely limit the morphological development parameters of *D. odorata*. Jiroutová et al. (2019) described a similar result with a species from the same family, *Pisum sativum*, where inhibitory effects of brassinosteroids on etiolated pea seedlings are mediated by an increase in ethylene production, and the minimum concentration of 24-EBL required to induce significant effects on ethylene production was approximately 20 nM.

Brassinosteroids do not act alone, but interact with other endogenous signaling molecules, especially the

phytohormones auxins, cytokinins, gibberellins, abscisic acid, ethylene, jasmonates, salicylic acid and strigolactones, forming complex signaling networks that modulate plant growth and development (Guo et al. 2024). They influence auxin signaling by modulating its polar transport, thus affecting processes such as lateral root formation (Nemhauser et al. 2004). In *Oryza sativa* (rice), auxin biosynthesis in lateral roots is nitrogen-dependent; under moderate nitrogen (N) deficiency, enhanced brassinosteroid signaling induces the expression of auxin biosynthesis genes, including tryptophan aminotransferase of Arabidopsis 1 (TAA1) and YUCCA5/7/8 (YUC5/7/8) via BZR1, thereby promoting increased auxin accumulation in the lateral root apical meristem and modulating lateral root elongation (Jia et al. 2021). Studies also indicate that brassinosteroids and gibberellins interact at the level of transcription factors, jointly influencing seed germination, cell elongation, apical hook development, resistance to zinc toxicity, and overall plant growth (Zhang et al. 2009; Ren et al. 2023). For *D. odorata*, the application of 30 nM and 40 nM of the hormone may have increased cell sensitivity to auxin and then promoted the effect of gibberellins. Therefore, these stimulated cell elongation and vascular differentiation, allowing the stem to grow in height and diameter. These hormones also seemed to have promoted phyllotaxy and then cell elongation, allowing for better-developed leaves.

Interactions between brassinosteroids and abscisic acid are also noteworthy, as abscisic acid can inhibit the brassinosteroid signaling pathway, and brassinolide (BL) can reduce ABA levels by suppressing the transcription of genes involved in ABA biosynthesis, suggesting cross-regulation between these hormones in the regulation of growth and adaptation to stress conditions (Goda et al. 2008; Ha et al. 2018). Brassinosteroids can also regulate ethylene biosynthesis, affecting phenomena such as fruit ripening (Hansen et al. 2009). Arteca et al. (1983) and Yi et al. (1999) demonstrated that brassinosteroids induce ethylene production, either alone or in synergy with other phytohormones, in etiolated mung bean seedlings (*Vigna radiata*). Moreover, ethylene is recognized as a cytokinin antagonist hormone (Stoynova-Bakalova et al. 2022), which plays a crucial role in nearly all aspects of plant growth and development, including cell division and differentiation, organogenesis, leaf senescence, the enhancement of leaf surface area, chloroplast formation, nutrient uptake, and stress responses (Li et al. 2021). The exogenous application of 24-epibrassinolide (EBL) increases cytokinin (CK) accumulation by inhibiting the transcription of the cytokinin oxidase gene 3 (CKX3), which is responsible for CK degradation (Yuldashev et al. 2012). This antagonistic effect of ethylene on cytokinin was manifested in this experiment by slight chlorosis observed in the leaves of treated plants, being more severe at a dose of 20 nM when compared to the control, which would explain the inhibitory effect observed in those plants.

Effects of Treatment on the Metabolic Characteristics of *Dipteryx odorata* Plants

Chlorophyll and nitrogen were two highly correlated metabolic variables and also the most important ones identified in this study. In addition, a decrease in leaf temperature was observed during the third and fourth months. Since chlorophyll measurement directly assesses photosynthesis by a plant and chlorophyll is produced from nitrogen and water (humidity) in the same plant, it is likely that 24-EBL may contribute to this strong correlation (Yu et al. 2023). By measuring the chlorophyll produced, we found that different concentrations of 24-EBL applied influenced photosynthesis in different ways. Wang et al. (2015) showed that the exogenous application of 24-EBL enhanced chlorophyll content in grapevine (*Vitis vinifera*) seedlings and, under water stress, also improved several photosynthetic parameters across all tested concentrations, including the effective quantum yield of Photosystem II (PSII), the maximum photochemical efficiency of PSII, maximum fluorescence, and the non-photochemical quenching coefficient of chloroplast thylakoid membranes. The 30 nM dose showed the best chlorophyll production in tonka bean seedlings, thus indirectly indicating the best photosynthesis achieved in the experiment, which would correspond to adequate nitrogen use. Akram et al. (2014) showed that *Jasminum sambac* plants treated with 3 µM of 24-EBL showed better results for growth indices, such as plant height, number of branches per plant, fresh weight, and dry weight of the flower. Similar studies have shown that brassinosteroids promote growth and increase the nitrogen content of *Ananas comosus* seedling shoots (Freitas 2010). Yu et al. (2023) also showed that exogenous application of 24-EBL plays a key role in improving nitrogen use efficiency in individuals, by increasing photosynthesis, the activities of carbon (C) and nitrogen (N) assimilation enzymes, nitrate absorption and transport, and the synchronized optimization of C and N distribution in seedlings. This explains the adequate growth observed in plants treated with 30 and 40 nM of hormone during this experiment.

In contrast, the 10 nM dose showed a slightly inhibitory effect after treatment, which was accentuated with the 20 nM dose by a considerable decrease in chlorophyll production, indicating possibly poor nitrogen utilization and metabolic stress. In *Hordeum vulgare* L. cv. Golden Promise, the exogenous application of EBL at high concentrations (1–10 µM) inhibits seedling elongation and root growth (Bajguz et al. 2019). Mechanistic evidence that brassinosteroids promote ethylene production was provided by Joo et al. (2006),

who showed that 24-EBL induces expression of the auxin-responsive 1-aminocyclopropane−1-carboxylic acid (ACC) synthase gene AtACS4 in *Arabidopsis* sp. This suggests a significant ethylene production at hormone doses of 10 nM to 20 nM. Stoynova-Bakalova et al. (2022) showed that endogenous or applied ethylene represses the promoting influence of cytokinin on cell division and cotyledon expansion in etiolated seedlings of *Arabidopsis thaliana*, which affects plant growth. Thus, the effect of ethylene, as opposed to that of cytokinin on shoot emergence, leaf surface improvement, and chloroplast formation, reduces the plant's ability to develop photosynthesis, which explains the decrease in leaf chlorophyll (Werner and Schmülling 2009; Mohorović et al. 2024).

In relation to the observed decrease in leaf temperature in all treatments, the highest rainfall occurred between months M1 and M4, and the lowest in months M0 and M5. For ambient temperature, the lowest averages were recorded between M1 and M3, while the highest occurred in M0 and M5. Perhaps more importantly, the historical average of daily sun hours is the lowest in March and April, corresponding to the experimental months M3 and M4, with March showing the highest number of rainy days. The combination of heavy rainfall, greater cloud coverage and lower temperatures during M3 and M4 may explain the reduction in plant leaf temperature observed in this experiment. Outside the M3–M4 period, the higher leaf temperature observed in the treated groups, compared with the controls, may be attributed to the effect of the hormone. Brassinosteroids are known to increase resistance to biotic and abiotic stresses, such as pathogens, extreme temperatures, saline soils, water scarcity, metal action, and nutritional deficiencies (Krishna et al. 2010; Bajguz et al. 2016). Studies have revealed a complex and diverse interaction between brassinosteroids, jasmonic acid, and salicylic acid, enabling plants to develop rapid and precise defense responses against agents responsible for various types of biotic stress, including microbial pathogens, viruses, insects, and necrotrophic or biotrophic organisms (Peres et al. 2019). Kurowska and Lazarević (2026) also reported that jasmonates, particularly jasmonic acid (JA) and methyl jasmonate (MeJA), constitute essential regulators of plant growth, development, and responses to abiotic stress, being widely recognized as stress-related hormones. They regulate key physiological processes such as leaf senescence, secondary metabolism, and nutrient homeostasis, thereby contributing to plant adaptation to drought and salinity. Methyl jasmonate (MeJA) enhances drought tolerance by promoting stomatal closure through ABA-dependent and ABA-independent pathways, improving root hydraulic conductivity, regulating stress-responsive genes, and activating antioxidant and osmotic adjustment mechanisms, thereby reducing water loss and oxidative damage under drought conditions (De Ollas and Dodd 2016). Their bioactive form, jasmonoyl-isoleucine (JA-Ile), activates signaling through the COI1-JAZ-MYC module, regulating the expression of genes involved in the balance between growth and defense, whereas OPDA may act as an independent signaling molecule. Furthermore, jasmonates tightly regulate leaf senescence by activating senescence-associated genes (SAGs) and modulating transcription factors such as MYC, WRKY, and NAC, in interaction with other phytohormones such as 24-EBL, leading to chlorophyll degradation, chloroplast disorganization, and global molecular reprogramming of plant cells.

Complementarity between Morphological and Metabolic Variables in *Dipteryx odorata* Plants

The use of 24-EBL to improve the growth and development of *D. odorata* plants influenced and strengthened correlations among variables. As the experiment was conducted in a semi-controlled environment with 50% shading and regular irrigation, the effect of the treatment was freely demonstrated through the data collected and then by the results obtained after analysis.

The results showed that, at various doses, 24-EBL influences the biological parameters of *D. odorata* plants in different ways. Starting with temperature, which was not correlated with any other variable in the experiment but varied according to the environmental conditions, we found that it did not influence the growth of the different plant groups in the experiment. De Oliveira and Lameira (2017) showed that normal water conditions associated with 80% light stress affected stomatal conductance in the species, which can limit carbon dioxide (CO_2) absorption and reduce photosynthesis. Thus, the transpiration rates of *D. odorata* plants would be affected, which could reduce growth by affecting the plants' biological responses. Coll et al. (2015) found that brassinosteroids are plant steroid compounds involved in many functions related to plant development, metabolism, signaling, and defense against a wide range of biotic and abiotic stresses. Therefore, we suggest that the application of 24-EBL likely improves the defense of *D. odorata* plants against stress caused by environmental temperatures. Under 50% shade, combined with an appropriate dose of hormone, the stomata may open to capture as much light and CO_2 as possible for photosynthesis, albeit generating a significant loss of water through transpiration due to the opening of the stomata which would require compensation through regular watering in order to ensure greater productivity.

This experiment suggested that applications of 30 and 40 nM of 24-EBL may have improved interactions between different endogenous hormones in *D. odorata* plants. One

possible mechanism that could explain such results is that the combined action of auxin, cytokinin, and gibberellins could allow for better responses in morphological parameters, including faster growth, increased stem diameter and a greater number of leaves, although further research is needed to elucidate the process. In addition, this combination could have contributed to the regulation of abscisic acid and ethylene, reducing physiological stress and resulting in improvements in metabolic parameters, such as greater nitrogen absorption and increased chlorophyll production. Aryal & Alferez (2025) showed that brassinosteroids interact with other hormones, such as auxins, gibberellins, ethylene, and abscisic acid, influencing all aspects of plant growth and development, acting on seed germination, vegetative and reproductive growth, photosynthetic efficiency, vascular differentiation, fruit yield and quality, as well as resilience to biotic and abiotic stresses. The 20 nM dose of 24-EBL showed an increased effect of morphological and metabolic stress, suggesting a possible hormonal imbalance associated with significant ethylene production. This response may be associated with the dose-dependent and non-linear nature of brassinosteroids (Jiroutová et al. 2019). Indeed, brassinosteroids are known to exhibit biphasic effects depending on the applied concentration, plant species, and physiological conditions. Liu et al. (2020) demonstrated that the same concentration of 24-epibrassinolide improved the tolerance of canola (*Brassica napus*) under high salinity conditions, while becoming detrimental under low salinity conditions, a phenomenon described as stress-level-dependent biphasic (SLDB) effects. Furthermore, Hafeez et al. (2021) reported that plant physiological and molecular responses are modulated by variations in endogenous and exogenous phytohormones, particularly brassinosteroids. Therefore, the growth inhibition effect observed at 20 nM may be associated with a transient metabolic adjustment or physiological inhibition caused by the sensitivity of *Dipteryx odorata* seedlings to this concentration, unlike the 10, 30, and 40 nM doses, which promoted morphophysiological development.

The present study demonstrated that the exogenous application of 24-epibrassinolide (24-EBL) significantly influences the morphological and metabolic development of young *Dipteryx odorata* plants cultivated under semi-controlled conditions with 50% shading. The responses were strongly dose-dependent, with 30 nM promoting the best overall performance by increasing plant height, stem diameter, leaf production, and chlorophyll accumulation, while 40 nM provided the best height/diameter ratio, indicating improved seedling quality and stability. In contrast, the 20 nM treatment induced inhibitory effects characterized by reduced growth, chlorophyll decline, and signs of physiological stress. These contrasting responses suggest that 24-EBL regulates plant development through a hormonal balance involving interactions with endogenous phytohormones.

The positive effects observed at 30–40 nM likely resulted from the optimization of auxin, cytokinin, and gibberellin mediated pathways, promoting cell elongation, vascular differentiation, nitrogen assimilation, and photosynthetic activity. Conversely, the inhibitory response at 20 nM may reflect excessive ethylene biosynthesis induced by brassinosteroid signaling, leading to metabolic imbalance and growth suppression. Beyond describing growth responses, this study highlights important physiological mechanisms underlying brassinosteroid action in an ecologically and economically important Amazonian species. These findings reinforce the potential of 24-EBL as a promising biotechnological tool to improve seedling production for reforestation and restoration programs in the Amazon, particularly by optimizing growth performance during early developmental stages. Further research may elucidate the hormonal and molecular mechanisms behind the inhibitory effects of 20 nM doses of 24-EBL in this species, particularly the potential involvement of ethylene overproduction, and help to ascertain an optimal dosage for large-scale seedling production.

Acknowledgments The authors thank the Institute for Forest and Biodiversity Development of the State of Pará (IDEFLOR-BIO) and the Federal University of Pará, Brazil, for the material support. KEvS thanks Alexandra Elbakyan, Ijad Madisch, Sören Hofmayer, and Horst Fickenscher for their contributions to science. EJHR would like to thank for the partial funding generously provided by the Brazilian Biodiversity Fund (FUNBIO) through the Floresta Viva initiative, and for financial support from the Brazilian National Bank for Economic and Social Development (BNDES) and Energisa, Fundo Vale, Norte Energia, KFW and Reset Assurini Project.

Author Contribution JS: Funding acquisition; Investigation; Resources; Writing – original draft. KEvS: Data curation; Formal analysis; Methodology; Validation; Visualization; Writing – original draft; Writing – review & editing. EHR: Funding acquisition; Project administration; Resources; Supervision.

Funding The Article Processing Charge (APC) for the publication of this research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (ROR identifier: 00x0ma614). This research was funded by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (Capes Foundation) of the Brazilian Ministry of Education under grant number 88887.146542/2025-00.

Declarations

Ethical Approval The authors certify that they have no affiliations or involvement with any organization or entity that has financial or non-financial interests in the subject matter or materials discussed in this manuscript.

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