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CO₂ Fertilizer Effect on Growth, Polyphenols, and Endophytes in Two *Baccharis* Species

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HIGHLIGHTS

- CO₂ fertilizer effect positively affected *Baccharis* primary productivity (PP).
- [CO₂] enrichment did not influence plant polyphenols and endophytes.
- Larger plants under [CO₂] enrichment presented greater polyphenol content.
- Changes in plants PP and secondary metabolites could affect community dynamics.

Abstract: In a climate change context, the buildup of CO₂ will affect plant communities worldwide. This study evaluated the effects of CO₂ enrichment on the development and defense of two Cerrado native species *Baccharis dracunculifolia* and *B. platypoda* and their associated endophytic fungi richness. The study took place in Open-Top Chambers, two with ambient CO₂ concentration (~400 ppm) and two in an enriched environment (~800 ppm). *Baccharis platypoda* developed 20% more leaves under enriched CO₂ conditions, whereas *B. dracunculifolia* was 30% taller and showed 27% more leaves than those under ambient conditions. In both species, leaf polyphenol concentration did not differ between treatments. Nevertheless, polyphenol content had a positive correlation with plant height on both species' individuals grown under CO₂ enriched conditions. Endophytic fungi richness and colonization rate on both plant species did not differ between ambient and enriched conditions. Our results show the positive effect of CO₂ fertilizer in at least one of the measured growth parameters. An important new finding was a synergistic increase in growth and chemical defense in both studied species under enriched CO₂ conditions, suggesting higher carbon assimilation and accumulation. This study suggests that the effects on primary productivity and secondary metabolites of *Baccharis* species will potentially reflect on the diversity and distribution of Cerrado plants and their associated animal communities.

Keywords: cerrado; climate change; carbon dioxide; functional homogenization; secondary metabolites.

INTRODUCTION

Fossil fuel combustion and deforestation are the main reasons underlying the unprecedented increase of carbon dioxide (CO₂) and other greenhouse gases (GHG) in the atmosphere [1]. In fact, annual CO₂ emissions from fossil fuel combustion alone increased from an average of 6.4 in the 1990s to 8.3 GtC (carbon gigatons) in the first decade of the 21st century [1]. Prior to the Industrial Revolution (ca. 1750), atmospheric CO₂ concentration was approximately 278 ppm, less than 300 years later, it reached an average of 400 ppm [1,2]. The latest IPCC Report estimates that at the end of this century, atmospheric CO₂eq (Carbon Dioxide Equivalent) concentration will reach 450 ppm on the Representative Concentration Pathways – RCP 2.6 scenario [3] (which assumes GHG emissions substantially declining after 2020) and >1000 ppm at the RCP8.5 (assuming emissions would continue to rise throughout the 21st century). Such concentration would translate in a temperature increase of approximately 1.5 °C on a very optimistic perspective (RCP2.6) and an alarming temperature of 3.7 – 4.8 °C in the pessimistic scenario (RCP8.5) [3]. These changes will substantially affect Earth's Systems and its organisms in many aspects such as through events of large-scale species extinction, functional homogenization as well as facilitating the success of species with invasive potential [4,5,6].

Regarding the effects of increased atmospheric CO₂ concentration on plant species, it is expected an overall increased growth, height, and number of leaves [7,8,9,10,11,12,13]. This phenomenon is called the "CO₂ fertilizer effect", which could be described as an increase in the photosynthesis rate of plants grown under such conditions [14,15,16]. In fact, C₃ plant species have been reported to present an increased growth of up to 50% [16,17] and the same effect has been observed for C₄ grass species after a long-term period [18].

There is evidence that cultivated species show increased photosynthesis rates and decreased protein concentration, leading to a nutritional quality loss of plant tissues under enriched CO₂ experimental conditions [8,14,19,20]. Additionally, by modifying the atmospheric C:N ratio, leaf nitrogen levels will decrease, causing the so-called "nitrogen dilution effect" observed in many studies [10,21,22]. Direct alterations in plants' physiology and biochemistry under high CO₂ conditions are also widely reported [23,24,25,26,27,28]. Alterations on resource allocation between primary and secondary metabolism could also affect plants' environmental plasticity, compromising their capacity to deal with environmental challenges [29,30,31,32,33]. In addition, such changes play a major role in plants associated organisms such as insects [19], potentially leading to impacts on the conservation and development of native [34], agricultural [35,36] and forestry species [36,37].

Endophytic fungi are associated with all plant species and play a decisive role in plant physiology and tolerance to harsh or simply atypical circumstances [38,39,40,41,42,43,44,45]. However, studies analyzing CO₂ increased effects on endophytic microbiota are scarce if we consider the overall potential of fungal symbionts on mitigating global change consequences [15,16,46,47]. In fact, fungal symbionts have shown to be particularly strong on mediating plant responses to climate change factors as drought and N deposition [47]. However, as the same study reported, there is a research gap specifically on leaf endophytes mediating CO₂ enrichment (only 3 out of 439 studies tackled this topic).

The genus *Baccharis* L. comprises about 440 species, one of the 10 most diverse genera of the Asteraceae family [48,49]. The genus is distributed from Canada to Southern Argentina and Chile [49,50] and includes fast-growing pioneer, such as *Baccharis platypoda* DC. and *Baccharis dracunculifolia* DC. These characteristics are well suited for the study of the effects of enriched atmospheric CO₂ conditions in species with wide spatial distribution [28]. In this study, we quantified the effect of increased CO₂ concentration in the physiology of *B. dracunculifolia* and *B. platypoda* and its consequences on these species' endophytic fungi richness. We aimed to test if (i) the carbon allocation in plant height and leaf number is higher under CO₂ enriched conditions; (ii) higher atmospheric CO₂ concentration stimulates plant secondary metabolism; (iii) an ambient enriched with CO₂ influence negatively the endophytic fungi richness and colonization rate; (iv) there is a negative correlation between polyphenol concentration and leaf endophytic fungi under CO₂ enriched conditions; and finally (v) enriched CO₂ environment impact the trade-off relationship between the growth and polyphenol concentration.

MATERIAL AND METHODS

Studied Species

Baccharis dracunculifolia and *Baccharis platypoda* are widely distributed shrubs in the tropical South American region [51]. *Baccharis* species' economical and biological importance is exemplified by their broad application, ranging from bioremediation to traditional medicine and cosmetic practices. The first is due to

their capacity to thrive in soils with wide nutritional variation and even in places with a high concentration of heavy metals [52,53,54,55,56]. The medical and cosmetic applications come from *Baccharis* high concentration of diterpenes, triterpenes and flavonoids [51,57,58,59,60]. Additionally, the species' potential to influence ecosystem functions further strengthens their importance for this study [28,61,62].

Establishment of seedlings and exposure to CO₂

To assess the effects of increasing CO₂ in both *Baccharis* species (Figure 1 b-c), we collected seeds from 15 individuals of *B. dracunculifolia* and 16 from *B. platypoda* in a rupestrian grassland area located at the Reserva Vellozia (19°16'46"S 43°35'13"W) in Serra do Cipó, Minas Gerais, Brazil. Seed collection was carried out in May/2009 for *B. dracunculifolia* and October/2009 for *B. platypoda*. These dates match the beginning and end of the rainy season, respectively, representing the *Baccharis* species flowering period [53].

The achenes were homogenized and stored at 4 °C. Afterward, seeds were planted in April of 2009 by separately placing 50 seeds of each species in 1.7 L high-density polyethylene pots (HDPE) with a 1:1 sand and vermiculite sterilized mixture. Growing procedures were carried out in four open-top chambers (OTCs) [63] under greenhouse conditions with 30% light intensity reduction screen in the Campus of the Federal University of Minas Gerais, in the city of Belo Horizonte, Minas Gerais, with an internal temperature between 25 °C to 35 °C [28].

Uniform seedlings were selected and pots were randomly distributed between the OTCs (Figure 1). Experimental variables (temperature and CO₂ concentration) were monitored using the Remote Integrated Control System - RICS 3.7 Evco, Italy software [28]. Two chambers received CO₂ injections twice the ambient concentration (750 to 800 ppm), while the other two chambers were exposed to ambient CO₂ concentration (approximately 390 ppm) [28]. All plants were watered on alternate days with 300 ml and pots were switched between each chamber of the same treatment in order to randomize exposure to CO₂ and other conditions.

Leaf Polyphenol and Endophytic Fungi Richness

Individuals without signs of pathogenic fungi were chosen for the evaluation of plant parameters and richness analysis. For *B. platypoda*, 34 individuals were placed in ambient condition chambers, and 31 in CO₂ enriched chambers (N= 65). For *B. dracunculifolia*, 25 individuals were grown under ambient setting, and 23 in chambers with enriched CO₂ conditions (N= 48). Both species seedlings were exposed to CO₂ conditions for 13 weeks, when data collection was performed. To estimate plant biomass in terms of carbon allocation, the plant height (from soil to the apex, cm) and leaf number per individual were measured. Consecutively, plant samples were bagged and taken immediately to the laboratory to perform the endophytic fungi analysis.

To quantify the endophytic fungi richness, three healthy mature leaves (without signs of herbivory or pathogens) were selected from every individual. Mature leaves were chosen due to greater endophytic fungi richness, and because of the absence of such microorganisms in young leaves of *B. dracunculifolia* reported by Oki and coauthors [64]. Each leaf surface was carefully sterilized following the protocol [65]: one minute in sterile distilled water; one minute in 70% ethanol solution; three minutes in 4% sodium hypochlorite solution; 30 seconds in 70% ethanol solution; and 90 seconds in sterile distilled water.

Subsequently, each leaf was cut into six 3 mm² fragments and placed in Petri dishes containing Potato-Dextrose-Agar culture medium and 250 mg L⁻¹ of Terramycin antibiotic to avoid bacteria growth [65]. Petri dishes were wrapped with plastic and kept at room temperature, in 12-hour photoperiod for 20 days [66,67]. Endophytic fungi grew radially to the leaf fragment after six days of incubation and were subsequently separated. Endophytic fungi isolated were morphotyped based on macroscopic morphological characteristics of the colonies as coloration (front and back), edge (smooth, lobed, wavy, distinct color from the center), texture (cottony, pulverulent, glabrous, creamy), and topography (high, flat, convex, umbilicate, cerebriform, rough) [68]. Isolated colonies were posteriorly submitted to microculture on glass slides to examine their reproductive structures, necessary for taxonomic identification [69]. However, the endophytes remained sterile, making it not possible to identify further than the recorded morphospecies.

Leaf polyphenol concentration was measured using a dual excitation fluorimeter (Dualex[®] 4.5 Scientific, Force One CNRS-Lure, France), a practical and quick tool for field measurements which evaluates the polyphenolic compounds in mature leaves, using data from ultraviolet absorption (UV) in the leaf epidermis [69,70,71,72,73]. The chlorophyll fluorescence emitted by UV light excitation is compared to the red-light excitation [69,70,71,72,73]. This method was chosen as it is nondestructive and leaves needed to be preserved for the endophytes analysis. In spite of its advantages, this method also has inherent limitations in the evaluation of the total quantitative content of phenolic compounds. Which is reasonable, considering there are more than 8,000 phenolic compounds described so far, from simple, low molecular weight

compounds and aromatic rings to large and complex tannins and derived polyphenols [74]. This limitation is particularly present when Dualex[®] is applied in comparisons among different species [75]. Nevertheless, in this study it was used for comparisons between treatments of the same species, thus, justified and efficient [70,71].

RESULTS

Effects of Elevated CO₂ Concentration on Plant Development and on Plant Secondary Metabolism

Carbon allocation pattern between ambient and enriched settings diverged within each plant species. More specifically, *B. platypoda* plants under enriched CO₂ condition did not show significant height difference when compared to the ambient condition group ($P > 0.05$, Figure 2a). Otherwise, they produced 20% more leaves when compared to individuals under ambient CO₂ concentration ($P < 0.01$, Figure 2b). *Baccharis dracunculifolia* individuals were on average 30% taller than individuals under ambient CO₂ condition ($P < 0.01$, Figure 2a). In addition, they presented on average 27% more leaves than the individuals in ambient chambers ($P < 0.01$, Figure 2b).

Leaf polyphenol concentration did not differ statistically between the CO₂ enriched and ambient condition for *B. dracunculifolia* ($P > 0.05$) and *B. platypoda* individuals ($P > 0.05$) (Fig. 2c). Nonetheless, larger individuals of both species had higher leaf polyphenol concentration (*B. platypoda* $r = 0.70$, $P < 0.001$; *B. dracunculifolia* $r = 0.63$, $P < 0.05$) based on the total leaf area for both species grown under CO₂ enriched conditions. Otherwise, this association was not observed in individuals grown in the ambient chambers (Figure 3; Table 1).

Table 1. Summary results for the effects of elevated CO₂ concentration on *Baccharis platypoda* and *B. dracunculifolia* development (height in cm, and leaf number), and estimated polyphenol concentration (molar extinction coefficient, $\epsilon = 20 \mu\text{mol cm}^{-2}$).

	Ambient condition	n	CO ₂ condition	n	P-value
	Height				
<i>B. platypoda</i>	1.30 ± 0.09	34	1.50 ± 0.19	31	$P > 0.05$
<i>B. dracunculifolia</i>	1.10 ± 0.20	25	1.70 ± 0.30	23	$P < 0.01$
	Number of Leaves				
<i>B. platypoda</i>	6.20 ± 0.16	34	7.20 ± 0.24	31	$P < 0.01$
<i>B. dracunculifolia</i>	5.40 ± 0.26	25	7.10 ± 0.23	23	$P < 0.01$
	Polyphenol				
<i>B. platypoda</i>	0.124 ± 0.006	12	0.136 ± 0.006	19	$P > 0.05$
<i>B. dracunculifolia</i>	0.109 ± 0.004	14	0.119 ± 0.004	14	$P > 0.05$

Parameters are represented by their mean values ± standard errors.

Effects of high CO₂ levels on endophytic fungi richness

In total, 26 colonies were isolated and classified into 20 fungi morphotypes (Figure 4). Ten fungi morphotaxa were recorded in *B. platypoda* grown in ambient chambers and three fungi morphotaxa from plants grown under CO₂ enriched condition. For *B. dracunculifolia*, four morphotaxa were reared from the individuals under ambient treatment while three fungi morphotaxa were reared from individuals grown under CO₂ enriched condition. There was no significant difference in endophytic richness between the ambient and enriched CO₂ treatment for both species ($P > 0.05$). Similarly, no statistically significant difference was found for the colonization rate in *B. platypoda* individuals grown under ambient condition (rate variation of 0 to 6.7%) and CO₂ enriched condition (rate variation of 0 to 3.3%) ($P > 0.05$). Likewise, no statistically significant difference was found for *B. dracunculifolia* individuals grown under the ambient condition (rate variation of 0 to 3.3%) and enriched CO₂ concentration (rate variation of 0 to 1.6%). Finally, there was no significant correlation between endophytic richness and polyphenol concentration ($P > 0.05$), as well as between ambient and CO₂ treatments for the two species ($P > 0.05$).

DISCUSSION

In the light of several studies on CO₂ “fertilizer effect” [7,8,9,11,12,13], plants under enriched CO₂ conditions may allocate a higher carbon content on their tissues and present a higher photosynthetic rate [28,77]. *Baccharis dracunculifolia* clearly showed higher growth and an increased number of leaves, probably

due to some traits that this species possesses which are associated with high growth potential. Some of them are great dispersion and establishment capacity and, most importantly, high conversion efficiency of atmospheric CO₂ into plant biomass (height and number of leaves) [28,61]. It is true that this capability is not usually sustainable once *B. dracunculifolia* reaches a certain maturity stage, mainly because of low nitrogen availability, typical of Cerrado soils [13,78]. Therefore, it is comprehensible that *B. dracunculifolia* would perform better than *B. platypoda* within the timeframe of this study [77,79,80,81]. However, even if limited to younger stages, the increase in height and leaf number reported here reinforces a very interesting discussion concerning light interception competition between herbaceous and woody species in Cerrado [13,84]. With increased aboveground photosynthetic material, woody species such as *Baccharis* shrubs would present higher light interception efficacy. According to the authors, this trend could ultimately result in a denser savanna, decreasing light availability to seedlings and specially to the herbaceous strata. Considering previous data on other Cerrado species [6,7,9,11,12,13,28] while adding to the discussion the results of both *Baccharis* species, further reinforces the potential magnitude that the CO₂ "fertilizer effect" could cause on the overall ecosystem functionality in the Cerrado, one of Brazilian hotspots [83].

Endophytic fungi richness and their colonization rate did not vary in accordance with polyphenol concentration, nor atmospheric CO₂ concentration. Little is known about the interaction mechanisms between endophytic fungi and their host plants. Therefore, predictions about the influence of different climatic factors - such as increased CO₂ - on endophytic fungi communities are at best anecdotal, in spite of some advances [10,15,84,85]. According to our microbiological analysis of both *Baccharis* species, we identified the presence of endophytic fungi in 15% of the leaf tissues of the sampled individuals. Consequently, the colonization rate and richness were also low. These findings are in accordance with a previous study at a nearby study site that reported eight endophyte fungus morphospecies in *B. dracunculifolia* [64]. It is likely that other carbon-based quantitative defenses, such as tannins and terpenes [86], commonly found in the *Baccharis* genus, but not examined in this study, might interfere with the relationship of endophytes and host plants in enriched CO₂ conditions. Otherwise, this is a much lower number of endophytes than found in Serra do Cipó - 58 morphospecies [87]. These contrasting results on endophytic richness and colonization rate are probably related to different ambient conditions characteristic to the sample locations. Therefore, it suggests the need for further investigation of the potential effects of environmental variations in endophytic fungi and the effects of increased CO₂ on them.

A strong positive correlation between polyphenol concentration and growth was also recorded for both native species exclusively under CO₂ enriched conditions. Polyphenols are responsible for a broad range of plant species physiological and performance roles [30,31,32,33]. Specifically, plants adaptability to environmental challenges such as light intensity, nutrient deficiency, low temperatures, and herbivore activity [32,88]. In this context, one of the most intriguing results of this study emerged from the finding of a positive relationship between plant height and their polyphenol content observed only under conditions of increased CO₂. The fact that the positive relationship was observed only on individuals grown under enriched CO₂ conditions may be explained through the growth-differentiation balance hypothesis, which affirms that photosynthesized carbon skeletons may be dynamically utilized for primary and secondary metabolites [29,33,89,90,91]. This dynamic follows the carbon trade-off principle, in which a series of functions compete among themselves [32,61]. Under the typical harsh conditions of Cerrado (e.g. water and nutrient low availability), plant species are characteristically of slow growth and have been selected to withstand such environmental circumstances by investing in defensive secondary metabolites and xerophilic morphology traits. However, in our experimental scenario of enriched CO₂ concentration, both species seem to overcome such allocation and environmental constraints, as taller plants also presented higher leaf polyphenol concentration.

Both *Baccharis* species showed a positive effect on CO₂ enriched conditions, by increasing their growth on at least one of the quantified parameters (height and/or the number of leaves). This represents an important achievement, especially when considering the typically slow growth pattern of the Cerrado plant species [13,61,78] and that their foliar polyphenol content increased concomitantly, contradicting the common trade-off found between these parameters [33,61].

CONCLUSION

Our study contributes to the knowledge about how two native Cerrado species, may respond in terms of growth, chemical defenses and effects on their endophytic fungi community in an ambient with enriched CO₂ concentration. Our results suggest that CO₂ increase exerts a positive effect on *B. platypoda* and *B. dracunculifolia* which can play a major role when considering the Cerrado species slow growth rates. Despite

the increased CO₂, it was not sufficient to affect the richness of endophytic fungi found in *Baccharis*. These facts indicate that in a scenario of increased CO₂ concentration, both *Baccharis* species and potentially several other Cerrado species, could reinforce challenges related to distribution range, outcompetition and functional homogenization. Consequently, it is imperative that further studies analyze atmospheric CO₂ effects, as well as other climatic variables on different species in an effort to contribute to the endorsement of more effective conservation practices.

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ERRATUM

ADDENDUM

As a complement for the material regarding the “ CO₂ Fertilizer Effect on Growth, Polyphenols, and Endophytes in Two Baccharis Species ” , with DOI number: <http://dx.doi.org/10.1590/1678-4324-2020190302> published in the journal Brazilian Archives of Biology and Technology, vol. 63, page 1-10, we are sending you the Figures below to be included.



Figure 1. a) Open-top chambers (OTC) used in this study; b) *Baccharis platypoda*; c) *Baccharis dracunculifolia*. Figures b-c by G.W. Fernandes.

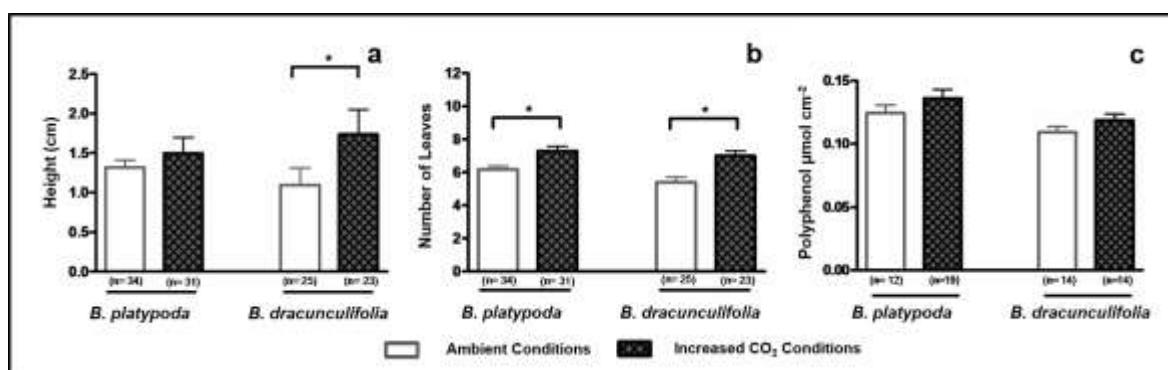


Figure 2. a) Plant height (mean + standard error), b) number of leaves (mean + standard error) and c) estimated polyphenol concentration ($\epsilon=20\mu\text{mol cm}^{-2}$) based on the total leaf area (mean + standard error) of intermediate leaves of *Baccharis platypoda* and *Baccharis dracunculifolia* grown under ambient and increased CO₂ conditions; *P<0.05.

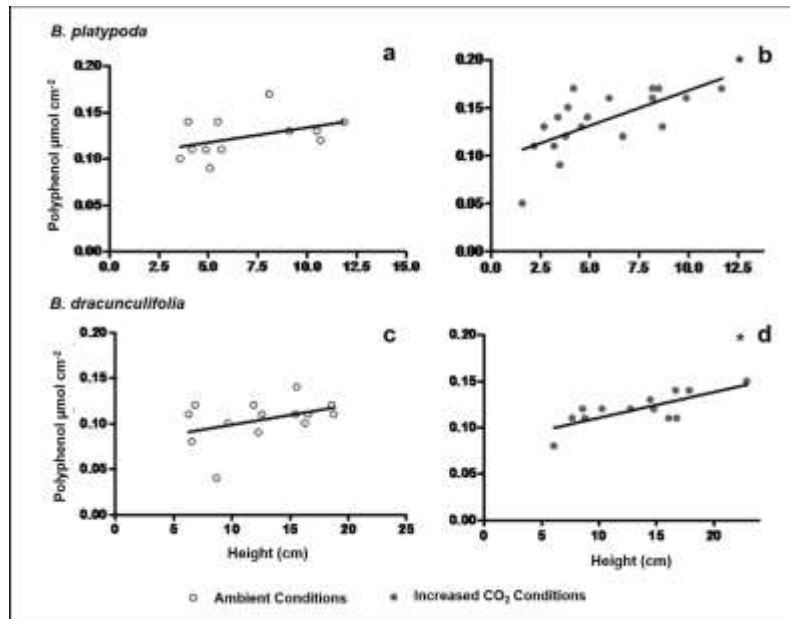


Figure 3. Plant height and polyphenol concentration based on total leaf area of *Baccharis platypoda* (a and b); and of *Baccharis dracunculifolia* (c and d). Empty and filled dots represent, respectively, ambient and increased CO₂ conditions *P<0.05



Figure 4. Some of the endophytic fungi morphotypes isolated from the *Baccharis* species.