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## Biometric and physiological responses of *Virola Surinamensis* to cadmium and biochar in amazonian soil

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The intensification of human activity, industrialization and urbanization has increased the risk of pollution due to large quantities of waste rich in heavy metals. Objective of this study was to assess the mitigating effects of biochar derived from açaí seeds on the biometric and physiological responses of *Virola surinamensis* plants grown in soil contaminated with cadmium. Seedlings of *V. surinamensis* grown in soils contaminated with varying doses of Cd (0, 10, 20, and 30 mg L<sup>-1</sup>) and different biochar proportions (0%, 5%, and 10%). Statistical analysis was performed using the multivariate exploratory principal components and the F-test, and when significant, the Tukey test was applied, and. At a dose of 10 mg L<sup>-1</sup> of Cd, the number of leaves was higher in the absence of biochar; however, this did not differ significantly from the treatment with 5% biochar, which was more effective in maintaining chlorophyll a content in the presence of Cd. Furthermore, for plants exposed to 10 and 20 mg L<sup>-1</sup> of Cd, the inclusion of 5% biochar mitigated the toxic effects of the metal, leading to increased rates of photosynthesis. Plants treated with 20 mg L<sup>-1</sup> of Cd also presented higher transpiration rates with 5% biochar application. For intercellular CO<sub>2</sub> concentrations, soils contaminated with 10 mg L<sup>-1</sup> of Cd demonstrated an increase in carbon concentration when treated with biochar. Thus, the addition of 5% biochar was effective in attenuating cadmium toxicity, suggesting its potential as a mitigation strategy for contaminated soils.

**Keywords** Biochar, Amazonian species, Heavy metal, Cadmium, Gas exchange, Ucuúba

The intensification of human activity, industrial activities and urbanization has increased the risk of pollution due to heavy metals alone. For example, mining produces large quantities of waste rich in heavy metals, such as cadmium, lead and zinc, in addition to destroying the vegetation cover of the area to be used, aggravating soil degradation, promoting water and wind erosion and leaching of pollutants into the groundwater, triggering a progressive degree of contamination of other areas<sup>1</sup>.

There are some bioremediation methods that are economically viable, environmentally friendly, and highly applicable<sup>2</sup>. Phytoremediation, for example, is one of the methods that can absorb metal compounds from the soil, such as cadmium, through special natural or deliberately bred plants using their roots to remove or reduce their bioavailability<sup>3,4</sup>. Phytoremediation includes plant extraction, plant stabilization, plant filtration, and plant stimulation<sup>5</sup>.

Phytoremediation of Cd-contaminated soil is a common method of Cd reduction. Hyperaccumulator plants generally have well-developed roots with the ability to adsorb high levels of heavy metals present in the soil and transfer them to above-ground parts<sup>6,7</sup>. To date, five species of Cd hyperaccumulator plants with high Cd

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detoxification capacity have been found. The Cd doses in their leaf tissue is generally greater than 300  $\mu\text{g g}^{-1}$  dry weight<sup>8</sup>.

Therefore, the use of plants in soil decontamination allows the valorization and conservation of species, for example *Virola surinamensis* (Rol. ex Rottb.) Warb plants, popularly known as ucuúba, an endangered forest species that grows relatively quickly, high biomass production, widely distributed and adapted to floodplain and igapó ecosystems in the Amazon, especially in estuaries. This Amazonian species has favorable characteristics as it is susceptible to contamination and important receptors for nutrients, organic and inorganic contaminants, including heavy metals. Therefore, it must have a certain tolerance to these metals, including cadmium and aluminum<sup>9</sup>.

*V. surinamensis* (Rol. ex Rottb.) Warb is a forest species with economic and medicinal interest, in addition to being useful for the restoration of altered areas. It is a widely distributed species and adapted to floodplain and igapó ecosystems in the Amazon<sup>10</sup>. These ecosystems are constantly susceptible to contamination by heavy metals<sup>11</sup>, indicating that the plant has strategies to tolerate environments contaminated by heavy metals.

The *V. surinamensis* plants has been the subject of studies as a way to conserve the native species and use it in degraded areas, seeking to evaluate its remedial potential by aiding in the decontamination of soils, including those contaminated with heavy metals, ensuring a balanced and sustainable environment. Furthermore, the research can serve as a basis for practical applications, whether industrial or agricultural, that seek to solve problems with waste or even ensure the income of traditional communities that depend on the resources offered by the species.

Among heavy metals, cadmium (Cd) is a highly toxic transition metal at very low exposure levels and has acute and chronic effects on the health of plants, animals, humans and all living beings. As a consequence of industrial activity and anthropogenic activity, it is estimated that 30.000 tons of Cd are released into the environment each year<sup>12</sup>. Cadmium is not degradable, so it persists in the environment once released. This property, together with its high mobility, bioaccumulative power and toxicity at very low concentrations, such as 0.003 mg L<sup>-1</sup>, make it one of the most important heavy metals<sup>13</sup>.

This metal is recognized as one of the most toxic and inhibitory of physiological processes in plants. Studies in several crops have shown that it reduces growth, photosynthetic activity, transpiration and chlorophyll content<sup>11,14-16</sup>. Furthermore, it causes chlorosis, oxidative stress, nutritional imbalances and modifies the activity of enzymes involved in the metabolism of organic acids and the Krebs cycle<sup>17-20</sup>.

In the Amazon, work with sustainable alternatives is being developed in favor of the reuse of waste, as is the case with the production of biochar from *Euterpe oleracea* Mart. (açaí) seeds. Biochar is a vegetable charcoal resulting from the pyrolysis process, capable of improving soil properties, water retention, preventing soil degradation and losses, increasing the content and sequestration of nutrients in the soil, mitigating the impact of potentially harmful substances toxic, promote the well-being of soil organisms; improve plant growth and biomass production and quality<sup>21,22</sup>.

Soil application of biochar-based composts can play a considerable role in environmental management and pollution reduction from mult-metal-contaminated soils<sup>23</sup>. Several studies have shown that biochar can potentially act as a soil pH regulator, carbon sink, and soil particle binder, as well as help stabilize long-term heavy metals in polluted soils<sup>24</sup>. In the latter case, the biochar in soils contaminated can promote the immobilization of metals, causing less absorption and phytotoxicity to plants<sup>25-27</sup>. According to Wang et al.<sup>28</sup>, biochar can reduce the mobility of metals in soils through the processes of ion exchange, specific adsorption and complexation improving the sorption capacity of the soil, the immobilization of the metal and, consequently, delaying the absorption of the metal by plants<sup>28-30</sup>.

Biochar has a direct influence on the bioavailability of compounds due to complexation, adsorption and precipitation processes, provided by its physicochemical characteristics (pH, ash, organic components and its porous structure) that help in the unavailability of contaminants<sup>31-33</sup>. However, despite the promising results on the biochar pyrolysis process and its influence on soil fertility and physical properties<sup>34,35</sup>, research on its applicability as a plant remediatior and attenuator, as well as its stability and regeneration in soils contaminated by heavy metals, requires further investigation in the Amazon region.

Considering the tolerance of *V. surinamensis* to cadmium, the high mobility of this metal and the potential of biochar in mitigating contaminated soils, we tested the hypothesis that açaí seed biochar influences the biometric and physiological characteristics of *V. surinamensis* plants exposed to areas contaminated by Cd. Therefore, the objective of this work was to evaluate the mitigating effect of *Euterpe oleracea* Mart. seed biochar on the biometric and physiological responses of *Virola surinamensis* plants subjected to doses of cadmium.

## Material and methods

The experiment was conducted in a greenhouse belonging to the Institute of Agricultural Sciences (ICA), of the Federal Rural University of the Amazon (UFRA) – Belém (PA), with geographic coordinates of 01° 27' 21"S, 48° 30' 16"W and average altitude of 10 m. The local climate is classified as Af, according to the Köppen classification, with an average annual rainfall of 3000 mm, an average temperature of 27 °C, an average humidity of 85% and high cloud cover throughout the year<sup>36</sup>. Controlling these variables is essential for plant development, maintaining the physiological activities of seedlings and protecting them against disease and pest attacks, in addition to preserving soil moisture and dynamics.

## Soil collection and preparation

Soil collection was carried out in a native forest area located inside UFRA, in the 0–20 cm depth layer, as it is the soil layer where the largest volume of roots of most cultivated plants is concentrated, we therefore seek to identify which nutrients are most available in the soil and whether they are in adequate concentrations for the plant used in this work<sup>37</sup>.

Depth	d	pH	OM	P	K <sup>+</sup>	Ca + Mg	Ca <sup>2+</sup>	Al <sup>3+</sup>	H <sup>+</sup> + Al <sup>3+</sup>	SB	CEC (pH7)	V*	m**
-	g cm <sup>-3</sup>	CaCl <sub>2</sub>	g kg <sup>-1</sup>	mg dm <sup>-3</sup>	mmol <sub>c</sub> dm <sup>-3</sup>							%	
0–20	2.65	3.5	29	6	0.6	6	4	18	133	7	140	5	72

**Table 1.** Chemical analysis of the soil in the 0–20 cm deep layer. pH = hydrogen potential; d = density; OM = organisational matter; P = phosphorus; K = potassium; Ca = calcium; Mg = magnesium; Al = aluminum; H = hydrogen; CEC = cation exchange capacity; SB = sum of bases; V\* = base saturation, m\*\* = aluminum saturation.

Layer	Clay	Sand	Silt	Textural classification
cm	g kg <sup>-1</sup>			SBCS*
0–20	212	693	95	Sandy Clay loam

**Table 2.** Particle size analysis of the soil in the 0–20 cm deep layer. \*Brazilian System of Soil Classification.

The soil was collected, dried in the open air, sieved through a 2 mm mesh and homogenized. During collection, a composite sample was taken for chemical characterization<sup>38</sup> and granulometric analysis<sup>39</sup>, which was subjected to soil analysis in the IBRA laboratory – Instituto Brasileiro de Análises, located in São Paulo. The results of these analyzes are presented in Tables 1 and 2, respectively.

After soil analysis, acidity correction was carried out using the base saturation method (V%) of soil, using 9.7 g of dolomitic limestone per pot (PRNT 97.4%) with the objective of reaching at least 50% base saturation as an indication of fertile soil, providing the necessary nutrients to the seedlings during the execution of the experiment. In addition to neutralizing, the soil acidity through the incorporation and reaction of calcium and magnesium for 30 days.

During this period, the soil remained in hermetically sealed plastic bags and inside the pots, where it was stirred every two days in order to homogenize the action of the limestone in the soil.

### Soil fertilization

After collection, the fertilization was carried out per pot according to the needs of the species and the chemical analysis of the soil in the EMBRAPA Recommendation Manual (2020). Macronutrients (166.66 mg of nitrogen, 170.45 mg of phosphorus and 0.103 mg of potassium) were used in the form of urea (45%), triple superphosphate and potassium chloride (52%); For micronutrients, fertilization was carried out for copper and manganese (8.40 mg of copper and 46.30 mg of manganese), as they presented low levels in the soil analysis, in the form of copper sulfate and manganese sulfate, respectively.

### Seedlings production

For the present work, *V. surinamensis* seedlings were used. These seedlings were produced from the germination of homogeneous and visibly healthy seeds, collected in March 2023 from mother trees located in the UFRA native forest area. In this process, polyethylene bags measuring 10 cm wide by 18 cm long were used, filled with soil collected in the 0–20 cm depth layer, where they remained in the nursery for 6 months due to their slow growth, characteristic of a late secondary or climax species, between the months of March to September 2023, being irrigated manually twice a day maintaining soil moisture.

After germination and development of the seedlings, 48 with uniform heights of approximately 20–30 cm, and healthy seedlings without the presence of visible deformations or pest attacks were selected to be used in the experiment.

### Soil exposure to cadmium chloride (CdCl<sub>2</sub>.H<sub>2</sub>O)

After the limestone reaction period, 3 kg of soil apparent density of 2.63 g cm<sup>-3</sup> was placed according to the pot's capacity (3 dm<sup>3</sup>). Contamination of the material occurred manually with the help of a beaker, where the solution was gradually placed in the soil at different doses of cadmium (Cd) (0, 10, 20 and 30 mg L<sup>-1</sup> of solution), in the form of cadmium chloride (CdCl<sub>2</sub>.H<sub>2</sub>O), considering agricultural, residential and industrial research reference values of 3, 8 and 20 mg L<sup>-1</sup>, respectively, presented in CONAMA resolution n°. 420 of December 28, 2009.

### Production, characterization and application of biochar

To produce biochar, açaí seeds were used, an agro-industrial material considered a residual byproduct of the açaí pulping process. The raw material was collected in local açaí beaters establishments, in the municipality of Belém, State of Pará. After collection, the seeds were washed in running water and dried in an oven at 70 °C for 24 h to ensure that the residues were completely dry. After drying, samples of 400 g of açaí seeds were prepared wrapped in aluminum foil and placed in a NOVUS – N1030 muffle oven with a heating time of 10 °C per minute, where they remained for 1 h at 600°C<sup>34</sup>, for the thermochemical conversion of biomass through the pyrolysis process. After this process, the size of the biochar particles was standardized using an electric mill and a sieve with a 2 mm mesh.

To characterize the biochar, all analyzes were carried out in triplicate, and the mean and standard deviation of the results obtained were calculated. The hydrogen potential (pH), specific surface area (SSA) and water retention capacity (WRC) followed the EMBRAPA methodology (2017).

From the immediate chemical analysis of the biochar produced, it was possible to determine the moisture (M), volatile material (VM), ashes (ASH) and fixed carbon (FC) (Table 3), following the methodology proposed in the ASTM standard D1762-84<sup>40</sup>.

The production of acai seed biochar and its application to the soil can improve aeration porosity, aggregate stability, and the proportion of soil macroaggregates. When biochar is produced at high temperatures, such as 600 and 700 °C, a material with high pH, greater recalcitrance, and greater potential for water retention in the soil is obtained, due to its hydrophilic character<sup>34,35</sup> and specific surface area.

### Incorporation of biochar into the soil

After correction and exposure of cadmium in the soil, the incorporation of biochar into the soil was carried out per pot, where it remained incubated for 60 days in different proportions, 0%, 5% and 10%, referring to the pot's capacity of 3 kg of soil, corresponding to 0 g, 150 g and 300 g, respectively.

According to the pre-defined biochar proportions, were the same ones used by Kul et al.<sup>41</sup> as soil mitigator, the material was weighed and incorporated manually with the help of a tray where charcoal and soil were mixed until a homogeneous and uniform mixture was obtained. At this stage, the turning was disregarded, because the improvements in soil quality resulting from biochar were affected by the soil disintegration process<sup>42</sup>.

### Experimental design

The experimental design used was randomized blocks in a  $4 \times 3$  factorial scheme, with four replications, totaling 48 experimental units. This factorial consisted of twelve treatments, with factor A corresponding to doses of Cd, one of them being the control treatment (without Cd), and the others with three doses of Cd (10 mg, 20 mg and 30 mg L<sup>-1</sup>); and factor B corresponds to the three proportions of biochar (0%, 5% and 10%).

### Biometrical variables

The measurement of biometric parameters was carried out upon removal from the experiment, 75 days after transplanting, when the plants showed visible symptoms of toxicity. Data were obtained on the following variables: aerial part height (H) measuring from the base to the stem apex, diameter at stem height (DSH), number of leaves (NL) and root length (RL).

The DSH was measured with the aid of a Digimess digital caliper, the length of the aerial part and the root were evaluated using a millimeter ruler, and the number of leaves per plant was calculated manually.

These variables are essential to monitor the development and growth of seedlings, seeking to analyze their behavior in contaminated soil and with biochar, and whether they are influenced by the different materials. Therefore, the plant material used in this study was identified by forestry engineers, researchers who are members of the Study Group on Biodiversity of Higher Plants. It is worth remembering that there were no voucher specimens of this material deposited in a publicly accessible herbarium.

### Photosynthetic pigments and gas exchange

Photosynthetic pigments (chlorophyll a, b and total) were determined in the leaf of the middle third of the plants in each experimental unit, with the aid of a digital chlorophyllometer ClorofiLOG model CFL 2060, brand Falker, in the morning between 09:00 and 10:00 h. The results obtained from the equipment are expressed as Falker Index (IF).

For gas exchange measurements, measurements were taken one day before removal from the experiment on expanded leaves in good phytosanitary condition, between 9:00 and 11:00 h, representing the daytime period of maximum photosynthesis. For measurement, a leaf from the middle third was selected in all experimental units of each treatment, using one block of plants at a time.

Each leaf was inserted and positioned in the median leaf region, inside the IRGA chamber, a photosynthesis meter, model LI-6400XT from LI-COR, Inc. Lincoln, coupled with a  $2 \times 3$  cm leaf chamber (Li6400-40) in concentration of CO<sub>2</sub> of 400  $\mu\text{mol m}^{-2}$  with an artificial light source with a flow of 1000  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , to determine the net CO<sub>2</sub> assimilation rate (A) ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), stomatal conductance (gs) ( $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), transpiration (E) ( $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), intercellular CO<sub>2</sub> concentration (Ci) ( $\mu\text{mol CO}_2 \text{ molar}^{-1}$ ) and internal/external carbon ratio (Ci/C<sub>a</sub>).

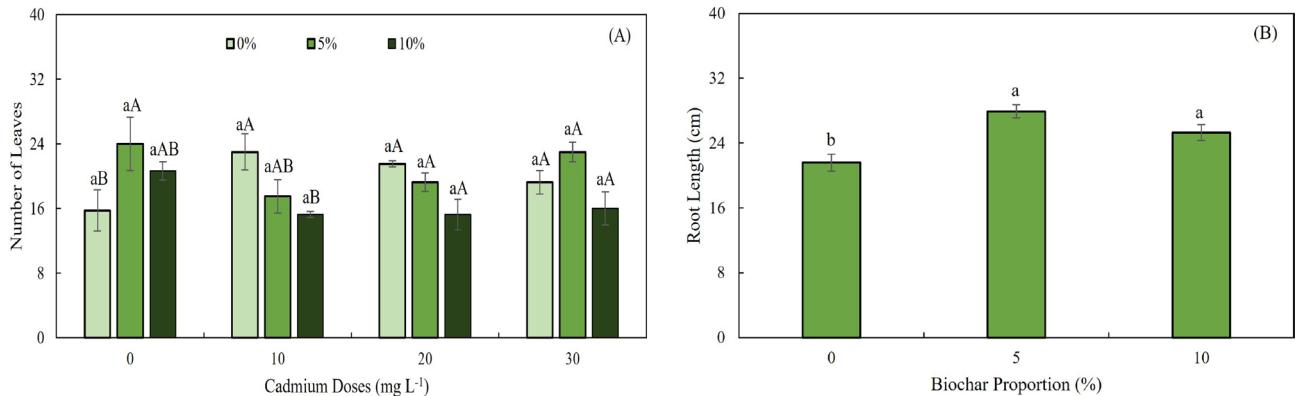
All variables mentioned had unique measurements on the day before the experiment was removed, individually and by seedlings, including all repetitions of treatments organized in randomized blocks.

Material	pH	SSA	WRC	M	VM	ASH	FC
	H <sub>2</sub> O	m <sup>2</sup> g <sup>-1</sup>	g g <sup>-1</sup>	%			
Biochar	11	340	2.5	9.2	0.5	98.4	1.1

**Table 3.** Chemical and physical characterization of BIO from *Euterpe oleracea* Mart. (açaí) seeds. pH = hydrogen potential; SSA = specific surface area; WRC = water relative content; M = moisture; VM = volatile material; ASH = ashes; FC = fixed carbon.

Source of variation	Test F												
	L.D	H	D	NL	RL	Chl a	Chl b	Chl Total	A	gs	E	Ci	Ci/Ca
Cadmium (Cd)	3	1.92 ns	1.74 ns	0.38 ns	0.65 ns	1.20 ns	1.34 ns	1.69 ns	16.57**	1.36 ns	6.78**	13.49**	7.67**
Biochar (BIO)	2	2.09 ns	0.58 ns	4.49*	11.86**	1.27 ns	2.22 ns	2.13 ns	25.43**	11.41**	5.94**	7.90**	1.69 ns
Cd x BIO	6	0.88 ns	1.93 ns	2.91*	1.90 ns	2.41*	7.53**	5.38**	24.77**	14.63**	3.40*	9.63*	13.64**
SEM	-	2.34	0.53	2.05	1.85	2.96	2.06	6.38	0.78	0.01	0.30	22.46	0.07
CV (%)	-	14.49	12.11	21.40	14.86	18.04	28.06	18.07	26.12	22.33	21.25	18.61	17.03

**Table 4.** Analysis of variance for height (H), diameter (D), number of leaves (NL), root length (RL), chlorophyll A (Chl a), chlorophyll B (Chl b), total chlorophyll (Chl total), photosynthesis (A), stomatal conductance (gs), transpiration (E), internal carbon (Ci) and relationship between internal and external CO<sub>2</sub> concentration (Ci/Ca) of *V. surinamensis* plants exposed to different doses of Cd and proportions of biochar. SEM: standard error of the mean; CV: coeficiente of variation; LD: liberty degree; ns: non significant; \*significant (p < 0.05); \*\*significant (p < 0.01) in the Tukey test for different Cd doses and biochar proportions.



**Fig. 1.** Number of leaves (NL) and root length (RL) (cm) in *V. surinamensis* plants exposed to different Cd doses and biochar proportions. Means followed by the same lowercase letter do not differ by the Tukey test (p < 0.05) between Cd doses in the same proportion of biochar, and means followed by the same capital letter do not differ by the Tukey test (p < 0.05) between the proportions of biochar at the same dose of Cd.

### Statistical analysis

For comparative purposes, the data obtained were subjected to analysis of variance using the F test using the statistical program AgroEstat version 5.4, and when significant, the means were compared using the Tukey test at a 5% probability level<sup>43</sup>. To verify the normality and homoscedasticity of data distribution, the Shapiro-Wilk test and the Bartlett test were performed, respectively. The mean values presented are followed by the standard error.

The data were also subjected to multivariate exploratory principal components and classification analysis using the hierarchical method and principal components performed in the program STATISTICA 7.0 (StatSoft. Inc., Tulsa, OK, EUA).

### Results

Among the variables analyzed and presented in Table 4, there was a significant interaction between the doses of Cd and the proportions of biochar for the number of leaves (p < 0.05), chlorophyll a (p < 0.05), chlorophyll b (p < 0.01), total chlorophyll (p < 0.01), photosynthesis (p < 0.01), stomatal conductance (p < 0.01), transpiration (E) (p < 0.05), internal carbon (Ci) (p < 0.05) and relationship between the internal and external proportion of CO<sub>2</sub> (p < 0.01). For the root length variable, there was a significant isolated effect only for the biochar proportions (p < 0.01).

### Biometrical variables

The NL in *V. surinamensis* plants was influenced by the interaction of biochar and Cd. In the absence of metal, plants subjected to 5% biochar showed higher NL (24 leaves), representing an increase of 52% (15.75 leaves). At a dose of 10 mg L<sup>-1</sup> of Cd, the NL was higher when biochar was not applied, however it did not differ from the treatment with 5%. For the other doses of Cd (20 mg L<sup>-1</sup> and 30 mg L<sup>-1</sup> of Cd) there was no significant difference for NL in relation to the different proportions of biochar (5% and 10%). Likewise, plants subjected to different doses of metal, without biochar, managed to maintain NL, becoming tolerant to the effects of the metal (Fig. 1A).

The RL of *V. surinamensis* plants showed a significant difference only for plants subjected to different proportions of biochar. The exposure of plants to charcoal, when compared to control (21.58 cm), provided an increase of 29.42% (27.93 cm) and 17.19% (25.29 cm) for RL in proportions of 5% and 10% biochar, respectively

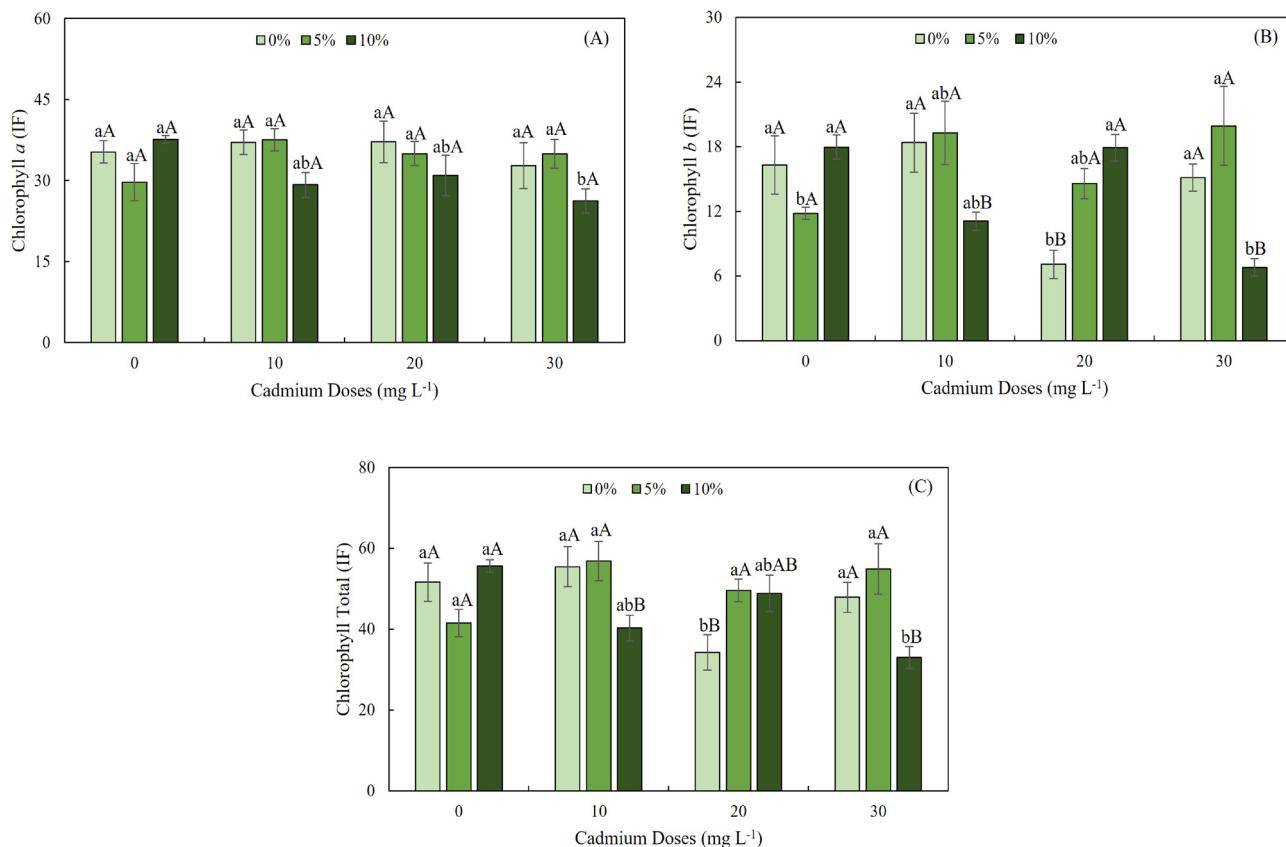
(Fig. 1B). Therefore, the effect of biochar in its highest proportion (10%) may have caused toxicity to the plants, affecting the length of the roots, which is why there was a 17.19% reduction in this treatment.

### Pigments

For the Chl *a* index, plants without Cd did not show a significant difference in Chl *a*, when exposed to different proportions of biochar. Likewise, plants exposed only to doses of the metal also showed the same behavior, characterizing a certain tolerance to the effect of Cd. However, the addition of biochar together with the doses of Cd significantly influenced the variable under analysis. In the presence of different doses of metal, 5% biochar was the proportion that managed to maintain the Chl *a* index in the plants. However, the 10% proportion reduced the amount of chlorophyll by 22.40%, 17.72% and 30.29% for doses of 10 mg L<sup>-1</sup>, 20 mg L<sup>-1</sup> and 30 mg L<sup>-1</sup> of Cd, respectively, showing a significant difference for the highest dose (30 mg L<sup>-1</sup> of Cd). Therefore, this proportion was not able to maintain or increase chlorophyll at this dose of Cd (Fig. 2A).

The Chl *b* index was lower in relation to Chl *a*. Plants subjected to metal-free treatments showed a significant difference in relation to the proportion of biochar, where plants exposed to 5% had a reduction of 28% (11.8) in Chl *b*, while the proportion of 10% biochar caused a 6% increase (17.96) in chlorophyll. At dosages of 10 mg L<sup>-1</sup> and 30 mg L<sup>-1</sup>, there was a significant difference when 10% biochar was applied, promoting a reduction in the chlorophyll index of 39.74% (11.07) and 55.09% (6.79), respectively. On the other hand, it was observed in plants with 20 mg L<sup>-1</sup> of Cd that the proportions of biochar improved the chlorophyll index by 106.10% (14.57) and 153.20% (17.90) in plants with 5% and 10% biochar, respectively (Fig. 2B).

For total chl, there was no significant difference for plants exposed to different proportions of biochar and no metal. In treatments with 10 mg L<sup>-1</sup> of Cd, the proportion of 10% biochar had a toxic effect on plants, reducing total chl by 27.11% (40.27). For plants exposed to 20 mg L<sup>-1</sup> of Cd, plants without biochar were affected by the metal, on the other hand, charcoal became effective in its different proportions, increasing by 44.65% (49.57) and 42.57% (48.86) the chlorophyll index referring to 5% and 10% biochar, respectively. At a dosage of 30 mg L<sup>-1</sup> of Cd, the plants showed similar behavior to those treated with 10 mg L<sup>-1</sup>, where the toxic effect of biochar (10%) also reduced the chlorophyll index (31.02%—33.02) (Fig. 2C).

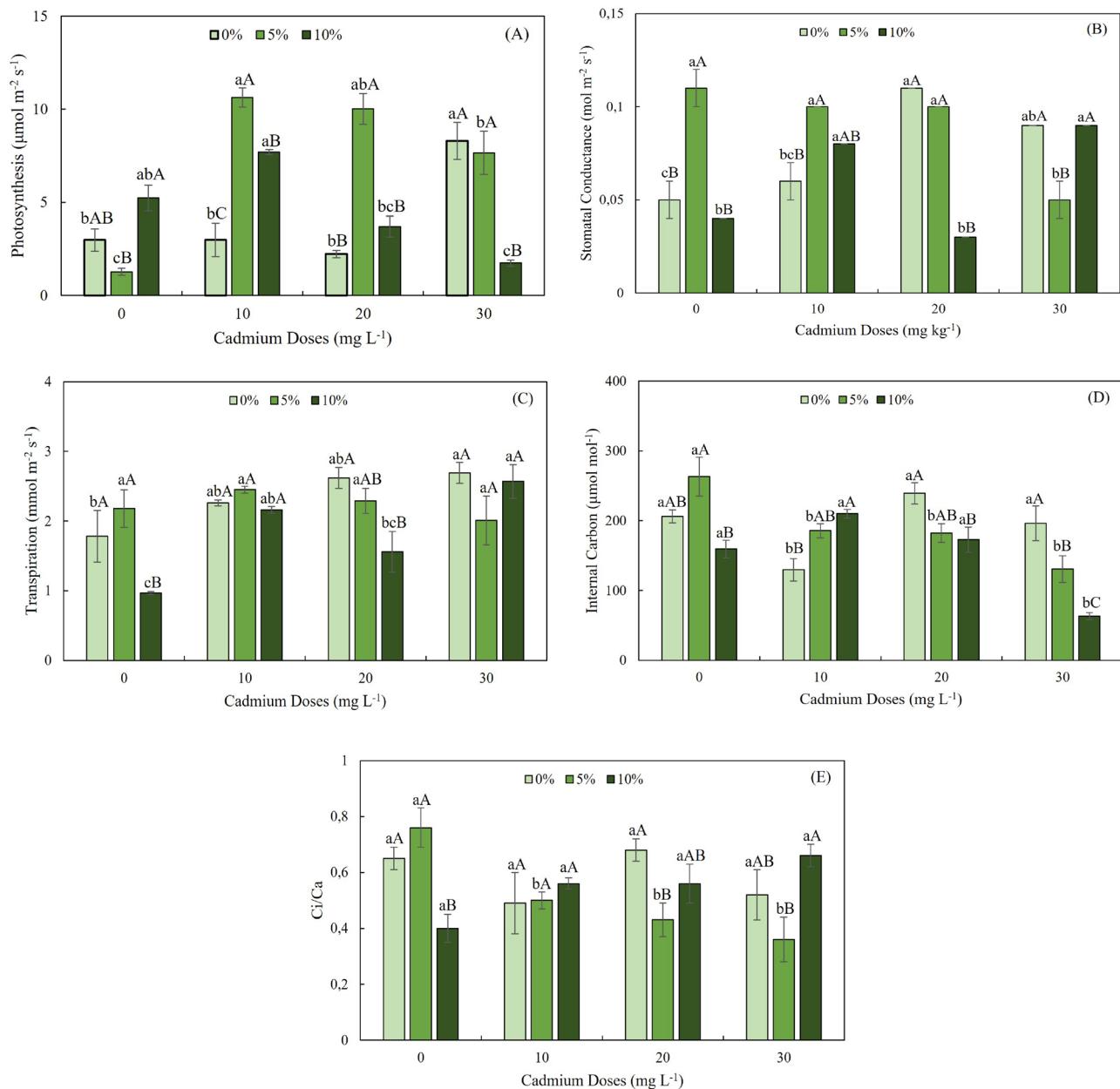


**Fig. 2.** Chlorophyll **a** (Chl *a*), **b** (Chl *b*) and total (Índice Falker—IF) in *V. surinamensis* plants exposed to different Cd doses and biochar proportions. Means followed by the same lowercase letter do not differ by the Tukey test ( $p < 0.05$ ) between Cd doses in the same proportion of biochar, and means followed by the same capital letter do not differ by the Tukey test ( $p < 0.05$ ) between the proportions of biochar at the same dose of Cd.

### Gas exchange

Analyzing  $A$ , there was a significant difference between plants exposed to different proportions of biochar and without metal, where 10% biochar provided an increase of 76% ( $5.24 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) in  $A$ . Plants subjected to  $10 \text{ mg L}^{-1}$  and  $20 \text{ mg L}^{-1}$  of Cd showed a significant difference between treatments with biochar, demonstrating similar behavior in plants with 5% biochar, mitigating the effect of the metal on the plant and increasing  $A$  by 256.71% (10.63) and 349.33% (10.02), respectively. The plants treated with  $30 \text{ mg L}^{-1}$  of Cd, when exposed to 5% biochar, were able to statistically maintain the  $A$  rate in the plants (Fig. 3A).

Analyzing the  $gs$  of *V. surinamensis* plants exposed to soils not contaminated by metal, 5% biochar provided an increase of 120% ( $0.11 \text{ mol m}^{-2} \text{s}^{-1}$ ) in conductance. Plants that received treatments with  $10 \text{ mg L}^{-1}$  of 5% Cd and 10% biochar showed an increase in  $gs$  of 66.67% ( $0.10 \text{ mol m}^{-2} \text{s}^{-1}$ ) and 33.33% ( $0.08 \text{ mol m}^{-2} \text{s}^{-1}$ ), respectively. In soil conditions contaminated with  $20 \text{ mg L}^{-1}$  of Cd, the addition of 5% biochar statistically maintained the conductance in plants, minimizing the possible toxic effects of the metal. This does not occur in the proportion of 10%, reducing this conductance to 72.73% ( $0.03 \text{ mol m}^{-2} \text{s}^{-1}$ ). Plants subjected to  $30 \text{ mg L}^{-1}$



**Fig. 3.** Photosynthesis (A), stomatal conductance ( $gs$ ), transpiration (E), internal carbon ( $Ci$ ) and Internal carbon and carbon relationship ( $Ci/Ca$ ) in *V. surinamensis* plants exposed to different Cd doses and biochar proportions. Means followed by the same lowercase letter do not differ by the Tukey test ( $p < 0.05$ ) between Cd doses in the same proportion of biochar, and means followed by the same capital letter do not differ by the Tukey test ( $p < 0.05$ ) between the proportions of biochar at the same dose of Cd.

of Cd showed similar behavior when exposed to 10% biochar, thus maintaining plant gas at the maximum dose of the metal used in this study. While plants at the same dosage, but with 5% biochar, were unable to achieve the same result, presenting a reduction of 44.44% ( $0.05 \text{ mol m}^{-2} \text{ s}^{-1}$ ) caused by factors that may have inhibited stomatal activity (Fig. 3B).

In treatments without metal, the proportion of 5% biochar provided an increase of 22.47% (2.18  $\text{mmol m}^{-2} \text{ s}^{-1}$ ) in the  $E$  rate of plants, in contrast, when exposed to 10% biochar, there was a reduction of 45.50% (0.97  $\text{mmol m}^{-2} \text{ s}^{-1}$ ) where the largest proportion may have had a toxic effect on the plants. This same behavior was observed in plants treated with  $20 \text{ mg L}^{-1}$  of Cd in different proportions of biochar. Plants exposed to doses of  $10 \text{ mg L}^{-1}$  and  $30 \text{ mg L}^{-1}$  of Cd did not show a significant difference in the  $E$  rate when biochar was incorporated. Likewise, the different doses of metal did not statistically influence the  $E$  rate in plants that did not receive biochar, presenting a potential tolerance of the species in relation to the metal (Fig. 3C).

In metal-free treatments, the proportion of 5% biochar provided an increase of 27.64% ( $263.20 \text{ } \mu\text{mol mol}^{-1}$ ) in the  $Ci$  of plants, on the other hand, when exposed to 10% biochar there was a reduction of 22.69% ( $159.41 \text{ } \mu\text{mol mol}^{-1}$ ), where the greater proportion of charcoal may have had a toxic effect on plants, as was also observed in *E. V. surinamensis* plants exposed to soils contaminated with  $10 \text{ mg L}^{-1}$  showed a significant difference for  $Ci$ , where the proportions of biochar managed to increase the carbon rate by 43.27% ( $185.75 \text{ } \mu\text{mol mol}^{-1}$ ) and 61.97% ( $210.00 \text{ } \mu\text{mol mol}^{-1}$ ) to 5% and 10% biochar, having a more representative effect in the largest proportion under study. However, plants subjected to doses of  $20 \text{ mg L}^{-1}$  and  $30 \text{ mg L}^{-1}$  of Cd showed a certain reduction in  $Ci$  of 23.78% ( $182.72 \text{ } \mu\text{mol mol}^{-1}$ ) and 27.78% ( $172.75 \text{ } \mu\text{mol mol}^{-1}$ ); 33.52% ( $130.54 \text{ } \mu\text{mol mol}^{-1}$ ) and 67.83% ( $63.17 \text{ } \mu\text{mol mol}^{-1}$ ), respectively, caused by the different proportions of biochar. With this, we can observe that at these dosages, the *V. surinamensis* plants managed to maintain the carbon rate in the absence of biochar, being the highest found among the treatments (Fig. 3D).

The  $Ci/Ca$  ratio showed significant differences in plants exposed to different proportions of biochar and without metal. In this condition, the proportion of 5% biochar caused an increase in the  $Ci/Ca$  ratio (16.92%– $0.76 \text{ mol mol}^{-1}$ ), while the highest proportion (10%) may have had a toxic effect on the plants, promoting a reduction of 38.46% ( $0.40 \text{ mol mol}^{-1}$ ), as was also observed in *E*. In the presence of the metal, the plants presented different behavior and the proportion of 10% biochar presented better results than the proportion of 5%. Plants exposed to  $10 \text{ mg L}^{-1}$  of Cd, regardless of the proportion of biochar, did not present statistical difference in the  $Ci/Ca$  ratio when compared to the control (0% biochar). When plants were subjected to soils contaminated with  $20 \text{ mg L}^{-1}$  and  $30 \text{ mg L}^{-1}$  of Cd, the 5% proportion reduced the  $Ci/Ca$  ratio to 36.76% ( $0.43 \text{ mol mol}^{-1}$ ) and 17.65% ( $0.56 \text{ mol mol}^{-1}$ ), respectively. However, the 10% biochar proportion was able to improve the activity of enzymes involved in  $\text{CO}_2$  fixation, resulting in high  $Ci/Ca$  values of plants exposed to  $30 \text{ mg L}^{-1}$  of Cd, representing an increase of 26.92% ( $0.66 \text{ mol mol}^{-1}$ ) (Fig. 3E).

The results of the principal component analysis generated for *V. surinamensis* plants with different proportions of biochar and cadmium dosages show an interrelation, explaining 60.28% of the total data variability. PC1 presented the highest percentage of variance, with 42.30%, while PC2, with 17.98%, obtained the lowest percentage of variance. In PC1, the variables height, Chl  $a$ , Chl  $b$  and Chl total presented positive loadings of 0.78, 0.91, 0.95, and 0.96, respectively, characterizing a direct correlation between the photosynthetic pigments. On the other hand, RL, gs,  $E$ ,  $Ci$ , and  $Ci/Ca$  presented negative correlation in PC1, with values of 0.30, 0.72, 0.57, 0.19, and 0.80, respectively.

In PC2, only the variables Chl  $a$  and  $Ci/Ca$  presented positive loadings of 0.04 and 0.25, respectively. The other variables presented negative correlation in PC2, mainly D, H, NL, A, gs,  $E$ , and  $Ci$ , with values of 0.81, 0.39, 0.66, 0.33, 0.57, 0.49, and 0.38, respectively. Furthermore, the biplot graph corroborates that the Cd30B5 ( $30 \text{ mg L}^{-1}$  of Cd and 5% biochar) treatment presented a higher correlation estimate, contrasting with Cd30B10 ( $30 \text{ mg L}^{-1}$  of Cd and 10% biochar), which presents a lower correlation estimate, between the variables Chl  $a$ , Chl  $b$ , total Chl and A, where the presence of 10% biochar was not efficient in mitigating the toxic effect on plants, confirming the hypothesis of this study (Fig. 4).

## Discussion

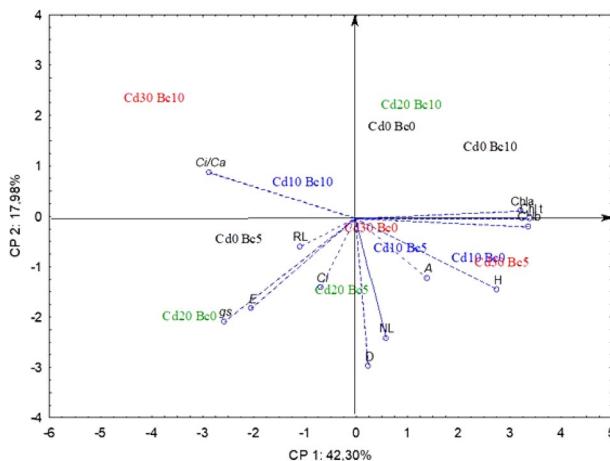
Among the biometric variables, the leaf was the main organ affected by Cd toxicity with  $10 \text{ mg L}^{-1}$  as seen in Fig. 1, since dosages higher than  $5 \text{ mg L}^{-1}$  can cause this toxicity, especially in young leaves due to its tendency to accumulate greater amounts of this metal<sup>44–46</sup>.

In this sense, the different proportions of biochar (5% and 10%) in Cd-contaminated soils managed to maintain NL in *V. surinamensis* plants, especially in treatments in which  $20 \text{ mg L}^{-1}$  and  $30 \text{ mg L}^{-1}$  of the metal were used, as shown in Fig. 1, because despite its toxicity, biochar tends to reduce the availability of metalloids<sup>47,48</sup> maintaining the biometric development of plants. Furthermore, studies carried out by Andrade Júnior<sup>10</sup> consider the species as tolerant to Cd, due to its ability to extract and accumulate metal in the root, restricting its transport to the aerial part, which could compromise the leaves by expressing its possible toxic effects.

For RL, the different proportions of biochar provided better root development by promoting nutrient absorption by plants, further minimizing the inhibitory effect of Cd stress<sup>49</sup> on cell division and root elongation rate cells which can be caused by irreversible cell obstruction<sup>50</sup>. However, some studies have shown that the cell wall can also increase plant tolerance to Cd by preventing the metal from entering root cells<sup>6,51,52</sup> through pectin, which is the main component of the cell wall that binds heavy metals<sup>51,53,54</sup>.

The application of biochar can increase the cation exchange capacity of the soil to retain and exchange these cations, thus increasing the availability of nutrients for plants<sup>55</sup>. With these benefits, consequently, biochar can improve soil fertility and reduce the availability of cadmium, thus improving soil, root growth and the rate of nutrient uptake of plants<sup>56</sup>, trying to maintain the biometric characteristics of plants.

Therefore, biochar may have influenced the absorption of essential nutrients and water for plant growth, as it has an alkaline pH, an ASE of  $340 \text{ m}^2 \text{ g}^{-1}$ , a RWC of  $2.5 \text{ g g}^{-1}$ , Cz content of 98.4% and CF of 1.1% as



**Fig. 4.** Principal component analysis of biometric and physiological parameters of *V. surinamensis* plants exposed to different Cd doses and biochar proportions. (NL)—number of leaves; (RL)—root length; (Chl a)—chlorophyll a; (Chl b)—chlorophyll b; (Chl t)—chlorophyll total; (A) – photosynthesis; (gs)—stomatal conductance; (E) – transpiration; (Ci) internal carbon; (Ci/Ca) internal carbon and carbon relationship; Cd0, Cd10, Cd20 and Cd30 represent the doses of cadmium; and Bc0, Bc5 and Bc10 represent the proportions of biochar.

shown in Table 1, improving the chemical, physical and biological characteristics of the soil. In addition to these characteristics, there are studies that show biochar as a beneficial product for the morphological development of the root, including an increase in root volume, surface area and root density, to acquire more nutrients and water to improve photosynthesis<sup>57,58</sup>, corroborating the results found in this work.

Furthermore, biochar has a strong binding effect on free Cd in the soil, being able to reduce the absorption of Cd by plants, its effective content in the soil, and consequently, minimize its stress effect on plant growth<sup>2</sup>, allowing the permanence of plant species in areas contaminated by heavy metals, as is the case of *V. surinamensis*, and can be used as an alternative in the recovery of these degraded areas.

Chl *a* is the most abundant pigment and plays a fundamental role in photosynthesis<sup>58,59</sup>. Its content is considered an indicator of damage to the photosynthetic system induced by environmental stressors such as heavy metals<sup>60</sup>, but even with 10 and 20 mg L<sup>-1</sup> of Cd, Chl *a* in *V. surinamensis* plants showed some tolerance to the metal, whether without or with 5% biochar.

On the other hand, plants subjected to 30 mg L<sup>-1</sup> of Cd, biochar (10%) were unable to maintain the Chl *a* content, reducing it as shown in Fig. 2A, since chloroplasts are the primary sites of action of the Cd and consequently, affect the biosynthesis of photosynthetic pigments, resulting in chlorosis, which appears as the most common symptom of metal toxicity<sup>47</sup>. Therefore, the results found for chlorophyll characterize the maximum dose of Cd as toxic, where the proportion of 10% biochar, as it is a material that provides essential micronutrients and macronutrients (nitrogen, phosphorus, calcium, potassium and zinc) to the soil<sup>61</sup>, may have provided an increase in nitrogen absorption, inducing a reduction in the enzymatic activity of chlorophyllase, and consequently, in the chlorophyll content<sup>62</sup>.

In Cd-contaminated soils, plants suffer abiotic stress and under this condition synthesize significantly less chlorophyll. This reduction in chlorophyll content leads to disturbances in the photosynthetic system and therefore chlorophyll is a suitable indicator of plant health decline<sup>63–65</sup>.

For Chl *b*, dosages of 10 mg L<sup>-1</sup> and 30 mg L<sup>-1</sup> of Cd did not statistically affect pigment production even without or with 5% biochar, since the plants showed a certain tolerance to the metal, guaranteeing the best Chl *b* index under these conditions (Fig. 2B). This proportion of biochar can alleviate the toxic effects of Cd stress on plant cells, protect the chloroplast structure of leaves, and increase the content of photosynthetic pigments<sup>2</sup>. Therefore, high levels of Chl *b* positively characterize the adaptability of the species due to the efficiency of photosystem II in carrying out photosynthesis<sup>59</sup>.

The incorporation of 5% biochar in soils contaminated with metal also provided the best total chl rate, due to the results of Chl *a* and Chl *b*, mitigating the toxic effect of the metal on *V. surinamensis* plants, with no significant difference between the different doses of Cd. Because the small particles of biochar improve its adsorption capacity due to the availability of additional sites effective in the adsorption of heavy metals, such as Cd<sup>66</sup>.

The total chl of plants subjected to doses of Cd and 10% biochar was lower when compared to 5% biochar as shown in Fig. 2C. This reduction can cause adverse effects on the photosynthetic system of *V. surinamensis* plants, affecting biosynthesis and the synthesis of complexes made up of chlorophyll, such as iron and zinc<sup>67</sup>. This micronutrient deficiency can also be caused by Cd toxicity, due to the ability to disaggregate chlorophyll-protein complexes that are combined or replaced by newly formed Cd-chlorophyll complexes. Therefore, the condition of the plant under stress reduces the expression of the iron citrate transporter in the xylem parenchyma cells, interrupting the flow of iron to the shoots<sup>68</sup>.

Therefore, the higher proportion of biochar (10%) may have caused toxicity to the plants' photosynthetic pigments, as its high ASE may have limited the release of nutrients to the plants, making it slower, causing them to become more adsorbed on the biochar surface.

The mitigating efficiency of biochar in photosynthetic activity is well known. Despite being a process highly sensitive to heavy metals<sup>46</sup>, charcoal at a proportion of 5% managed to maintain and improve A's performance due to its ability to capture and store carbon<sup>69</sup>. This directly influences plant growth due to its relationship with the carbon cycle, and when absorbed, it is stored and incorporated into the plant structure, integrating organic compounds<sup>70</sup>.

Studies conducted by Ali et al.<sup>3</sup> state that the addition of biochar improves the accumulation of nitrogen in the soil and its content in the leaves, increasing the photosynthetic rate. This occurs due to the improvement in the physicochemical properties of the soil, which ultimately increases the accumulation of nitrogen and, consequently, the photosynthetic activity<sup>58,71,72</sup>.

Although the condition of the sandy clay soil together with biochar may have a slow release of nutrients, those that were released during the plant's stay in the soil were sufficient to absorb water and nutrients that improved the photosynthetic apparatus. Possibly the amount of chlorophyll present in this plant was sufficient to absorb light and carry out the photosynthetic process properly.

On the other hand, increasing the proportion of biochar (10%) did not present the same result, since charcoal was unable to mitigate the toxic effects of Cd, considered an effective inhibitor of A<sup>73</sup> which may have limited nitrogen accumulation by immobilizing it in *V. surinamensis* plants due to the high C/N ratio seen in Fig. 3A<sup>74</sup>. Its mode of action includes disruption of chloroplast ultrastructure, inhibition of Calvin cycle enzymes, chlorophyll biosynthesis and reduction in CO<sub>2</sub> assimilation due to stomatal closure<sup>36,75–77</sup>.

Even under toxic conditions (10 and 20 mg L<sup>-1</sup> of Cd), *V. surinamensis* plants were able to maintain their gs when exposed to 5% biochar. Thus, the reduction of gs would result in low mesophilic conductance to CO<sub>2</sub> and consequently in lower chloroplast CO<sub>2</sub>, justifying the decrease in photosynthesis in *V. surinamensis*, found in the research carried out by Andrade Júnior<sup>10</sup>. However, for 30 mg L<sup>-1</sup> of Cd, this proportion was not effective, reducing the conductance caused by the plant's contact with Cd, and the metal, upon entering the guard cells, is responsible for closing the stomata<sup>45,78</sup>, then stomatal resistance increases<sup>17</sup> as a tolerance strategy to reduce the absorption of heavy metals from the soil, their accumulation and their phytotoxic effects<sup>10</sup>.

These factors allow it to maintain its physiological activities, since biochar, even at the maximum proportion used in this study (10%), will not always be able to maintain or improve the gs of plants subjected to different dosages, as seen in Fig. 3B at 20 mg L<sup>-1</sup> of Cd. It is also worth mentioning that this efficiency can be influenced by the metal due to its mobility, as is the case with Cd, used in the research by Nogueira et al.<sup>79</sup> with *Schizolobium amazonicum* Huber (Paricá), a forest species sensitive to applied doses of cadmium, interfering with gas exchange and plant growth.

In soils contaminated with 10 mg L<sup>-1</sup> and 30 mg L<sup>-1</sup> of Cd, plants were able to maintain their E rate, even when exposed to 5% or 10% biochar. This did not occur with plants exposed to 20 mg L<sup>-1</sup> of Cd with 10% biochar, showing a reduction in E due to the ability of the metal to minimize the partial pressure of CO<sub>2</sub> in the stomata, and consequently, in the gs<sup>80</sup> of *V. surinamensis* plants. This reduction is caused by the effect of Cd in compromising the water status and altering the permeability of the plasma membrane<sup>81</sup> of plant tissues in plants under stress conditions, which induces cellular vacuolization, narrowing of the intracellular space, reduction in the number of chloroplasts and increase in cell size, limiting water content and gs<sup>82</sup>.

Taking Ci into account, different doses of Cd reduced the carbon content when plants were exposed to 5% biochar. Since charcoal was unable to minimize the toxic effects of the metal, there was a reduction in the electron transport capacity in the leaves, and consequently, in the assimilation of Ci, which is possibly associated with damage to the thylakoids<sup>83</sup> replacing compounds from the chlorophyll metabolic pathway<sup>84,85</sup>.

However, the stomatal opening can reverse this situation by contributing to the entry of CO<sub>2</sub> into the mesophyll of the leaves, increasing the internal proportion of this gas, which may have happened in plants treated with 10 mg L<sup>-1</sup> of Cd and 10% of biochar observed in Fig. 3D, causing an increase in transpiration<sup>67</sup>.

Taking into account the Ci/Ca ratio in *V. surinamensis* plants, this variable was more efficient when there was the incorporation of 10% of biochar in soils, demonstrating greater CO<sub>2</sub> fixation. This may be the result of biochar's ability to bind to heavy metals due to its adsorbent properties, reducing their availability and mobility in the soil<sup>50,86</sup>, otherwise they would be more accessible and easily transported within cells, damaging this relationship of CO<sub>2</sub>.

Furthermore, açaí seed biochar is capable of assisting these gas exchanges with the environment (A, E and gs) without compromising the Ci/Ca concentration<sup>87</sup>, presenting good adsorption capacity for several heavy metal ions<sup>3,88</sup>. Thus, its chemical or physical activation significantly increases the adsorption capacity for heavy metals<sup>89</sup>, such as Cd, limiting its translocation to plant organs and its effects on metabolic pathways, which may have stimulated the occurrence of the Calvin Cycle, for example, increasing the activity of Rubisco, and consequently, the carbon fixation capacity<sup>90</sup>.

The incorporation of biochar minimizes the availability, accumulation, and toxicity of Cd in plants<sup>91,92</sup>, avoiding disruptions in the metabolic pathways of plants. And when combined with fertilization, Ahmad et al.<sup>93</sup> and Dharma-Wardana<sup>94</sup> state that essential plant nutrients have direct and indirect effects on these Cd conditions. For example, nutrients can minimize the solubility of the metal in the soil, facilitating several mechanisms, including: Cd sequestration in vegetative parts, adsorption and precipitation, competition between plant nutrients and metal for the same membrane transporter, and preventing metal accumulation in seeds. Likewise, indirectly, these nutrients can promote Cd dilution, relieving plant physiological stress and improving its productivity<sup>95</sup>.

It is worth remembering that plants also have defense mechanisms in response to abiotic stress conditions, reducing the accumulation of metal in plant cells and tissues due to the low intake and deposition of these

elements in the roots, even though they are the first organ of the plant to come into contact with the metal<sup>96–98</sup>, making them the most effective preventive form in the absorption of heavy metals by plants<sup>68</sup>.

According to the biplot graph presented, resulting from the analysis of the principal components, the variables photosynthetic pigments, gs and Ci/Ca stood out due to their correlation and functionality in the presence of Cd and biochar, especially when the proportion of 5% can mitigate the effect of metal toxicity, associated with the potential of the species to tolerate soils contaminated with Cd, as seen in the study carried out by Andrade Júnior<sup>10</sup>. Plants can increase soil pH through their root exudates that bind to metals and precipitate them in the apoplast, blocking their entry into the cell<sup>99</sup>, which may have ensured the greatest production of photosynthesis, reaffirming the results of Fig. 3A in accordance with Fig. 4, allowing their development in environments contaminated by Cd, until further work can be developed with other heavy metals.

## Conclusion

The use of biochar helped the biometric and physiological development of *Virola surinamensis* (Rol.) Warb plants exposed to cadmium. However, analyzing the set of variables under study, despite the proportion of 10% biochar having presented a toxic effect on total Chl, A and Ci/Ca at 30 mg L<sup>-1</sup> of cadmium, and maintaining some results found for NF, RL, E and gs at 5% biochar, mainly at the doses of 10 and 30 mg L<sup>-1</sup> of cadmium, this proportion (5%) was more efficient in attenuating the toxic effect of the metal and managed to maintain or improve the behavior of the plants subjected to cadmium. Therefore, the use of 5% biochar is considered recommended for mitigating soils contaminated by cadmium, however, field studies must be carried out to validate the efficiency of biochar at this proportion.

In the principal component analysis, it was found that Cd negatively affects the biometric and physiological characteristics of *V. surinamensis* plants, mainly chlorophyll production and photosynthetic activity. In addition, it is possible to corroborate the hypothesis under study, that biochar helps to reduce the toxic effects of the metal, mainly in the proportion of 5%, since the 10% biochar did not present the same expected effects, although some variables showed a positive response.

In addition, the behavior and importance of *V. surinamensis* plants in timber, medicinal and industrial applications make it a potential species for studies on soil remediation and recovery of degraded areas, leading to the search for new research to be carried out using 5% biochar or even different biochars. This bioproduct used in this work ensures the importance of reusing waste and applying it to the soil as a mitigating factor, bringing environmental, social and economic gains that involve agricultural practices, economic dynamics and an effect on climate change, as it is considered a carbon stock.

## Declaration

We certify that the doctoral student and her advisor had permission and license to collect seeds of *Virola surinamensis* at the Institute of Agricultural Sciences of the Federal Rural University of the Amazon, as well as to conduct her experiment for her doctoral thesis at the Institute, comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

## Data availability

The data generated in this research is in the hands of the person responsible for the research, Dayse Gonzaga Braga, who is available to provide any data that the evaluation process suggests, without any objection, the relevant data has not yet been placed on any platform.

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## Author contributions

DGB: responsible for administering the experiment and writing the text. RLCF: responsible for contextualizing and writing the text. CBS and JACC: conducted the experiment. ACBA: performed the statistical analysis, tables and figures. AEAB: responsible for supervising and validating the data. VRN: responsible for translating the text. LCS: responsible for editing the text. CFON: responsible for the analyses performed. All reviewed the manuscript.

## Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

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