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Foliar Structural Aspects and Essential Oil Yield of Two *Croton* L. Species (Euphorbiaceae) from the Amazon

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Abstract

Croton cajucara Benth. and *C. sacaquinha* Croizat are known as "sacaca" and "sacaquinha", respectively in the Amazon region. Both species are used in Amazon folk medicine for their pharmacological potential, associated mainly with rich phytochemical properties of leaves and essential oil. Aiming to identify and determine the number of secretory structures related to essential oil yield, leaves were harvested in the medicinal plant garden of Embrapa Amazonia Oriental, Belem, Pará, Brazil, and were submitted to microscopy techniques and essential oil extraction. The species presented differences in trichome types and frequency, as well as essential oil yield. Furthermore, a positive relationship between frequency of secretory trichomes and essential oil yield was observed. The results obtained highlight the potential of *Croton* species to provide oils and could represent a viable alternative for sustainable exploration and a source of income for Amazonian communities.

Keywords: Non-woody forest products, secretory structures, ultrastructure.

1. INTRODUCTION AND OBJECTIVES

Croton L. (Euphorbiaceae) species are recognized in Brazil for their ornamental and pharmacological potential. Investigations into the foliar structures of *Croton* commenced in the mid-20th century (Metcalfe & Chalk, 1950). Early studies identified characteristic structural aspects, including stellate pubescence, glandular structures on leaf margins, bases, apices, and petioles, as well as tector, lepidote, and dendritic trichomes, which exhibited a random distribution across the leaf surface. Since these early investigations, further anatomical studies have been conducted on different *Croton* species, significantly advancing the understanding of the genus taxonomy (Riina et al., 2015, Rosa et al., 2021, Vitarelli et al., 2015). More recently, detailed studies on trichome classification within *Croton* have highlighted the considerable diversity of these structures, emphasizing their functional and taxonomic significance (Pinto-Silva et al., 2023).

Two *Croton* species with occurrence in the Amazon region, *C. cajucara* and *C. sacaquinha*, are recognized in folk medicine as "sacaca" (Conceição et al., 2016, Caruzo et al., 2023). These species are characterized by glandular trichomes presence, which in *C. cajucara* serve as reservoirs for essential oils (Pinto-Silva et al., 2023, Mendonça et al., 2008). *Croton cajucara* exhibits morphological variation, with two distinct morphotypes: «common leaf» and «red leaf.» These morphotypes differ in their phytochemical profiles, demonstrating differences in bioactive compound concentration (Chaves et al., 2006). The essential oil extracted from the trichomes of *C. cajucara* is a complex mixture of compounds, including linalool (Camargo & Vasconcelos, 2014; Sobrinho et al., 1998), aromadendrene, germacrene D, bicyclo germacrene, α-muurolene, γ-cadinene, δ-cadinene, germacrene B, spathulenol, caryophyllene oxide, dill apiole, τ-cadinol, α-cadinol, and 7-hydroxycalamenene (Azevedo et al., 2021).

Given the full potential of sacaca essential oil, it has been suggested that it has the potential to replace essential oil extracted from *Aniba rosaeodora* Ducke, popularly known as rosewood, which has been overexploited and consequently is under threat of extinction (Souza et al., 2003, Mendonça et al., 2008). The documented antimicrobial and antifungal properties of *C. cajucara* essential oil (Alviano et al., 2005, Azevedo et al., 2012, Azevedo et al., 2021) suggest its potential application as a natural preservative on plant-based products. Additionally, due to these properties, the leaf extracts of this Croton species could also be tested in the phytosanitary control of seeds (Carvalho et al., 2022), and as a biopesticide within organic and sustainable agricultural practices (Gulzar et al., 2021).

Community-based forest management of non-timber forest products in the Amazon, particularly those related to cosmetic production, is recognized as a viable strategy for augmenting household income in the region (Antunes et al., 2021). Considering the renewable nature of leaves, the bioactive potential of *Croton* species and morphotypes found in the Amazon could significantly contribute to the bioeconomy, thereby enhancing the livelihoods of Amazonian communities and preserving forest ecosystems (Alcântara et al., 2010, Lopes et al., 2018, Shanley & Medina, 2005).

Although previous studies have investigated the chemical composition of leaf extracts from *C. cajucara* and *C. sacaquinha*, there is limited information regarding the secretory structures in these species, particularly in terms of their anatomy, abundance, and association with essential oil production. Moreover, it is well-established that the location, density, and function of leaf trichomes within the *Croton* genus exhibit considerable variation (Pinto-Silva et al., 2023). Therefore, the objective of this study was to characterize the leaves of *C. sacaquinha* and two morphotypes of *C. cajucara* concerning their anatomical and chemical properties. Specifically, the study focused on: (I) the identification, comparison, and quantification of secretory structures, and (II) the determination of essential oil yield. This research aims to enhance the understanding of these Amazonian species, especially those of the *Croton* genus, and their potential for the valorization of non-woody forest products.

2. MATERIALS AND METHODS

2.1. Site and material characterization

The material used in these analyses was harvested from three plants per species/morphotype with approximately eight years at the Horto de Plantas Medicinais (Medicinal Plant Garden) of Embrapa Amazônia Oriental, located in the city of Belém, between August and November 2016, always between 10 and 11 am. All individuals had grown in an understory environment with sandy soil. Throughout the harvesting months, the average temperature was 27 ºC, accompanied by a mean relative air humidity of 77% and an average rainfall of 108 mm (Instituto Nacional de Meteorologia, 2023).

Croton cajucara, "red leaf morphotype" (Figure 1A) presents dark-green, simple, alternating, petiolated, pinnate venation leaves, with a membranous aspect, hairy, with oblong-lanceolate leaf blade with an entire margin, cuspidate to slightly caudate apex, obtuse base, with stipules at the leaf base; mean length of leaf blade of 14.79 cm and mean width 4.43 cm (Figure 1D). The "common leaf" morphotype (Figure 1B) has light-green simple, alternating, petiolated leaves, with pinnate veins, leaf hairy surface, with elliptical-lanceolate shape, entire margin, cuspidate apex and obtuse base. Mean leaf length and width were 9.82 and 3.51 cm, respectively (Figure 1E). Leaves of *Croton sacaquinha* (Figure 1C) are simple, alternating, petiolated, with pinnate venation, membranous aspect, hairy surface, with leaf blade lanceolate shape, entire margin, acute to acuminate apex, obtuse base, presenting interpetiolar stipules with 7.75 in length and 1.38 cm in width (Figure 1F).

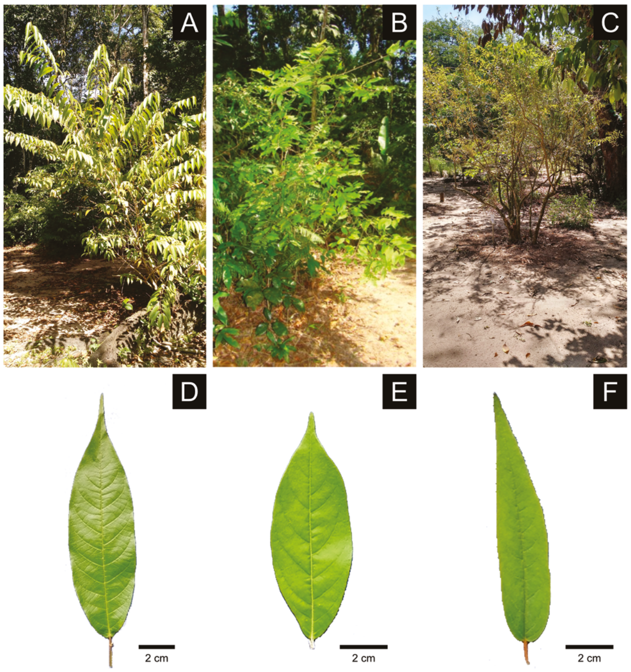

Figure 1. *Croton cajucara* L. morphotypes: "red leaf" (A) and (D); "common leaf" (B) and (E); *C. sacaquinha* Croizat: (C) and (F).

2.2. Anatomical and histochemical analysis

Leaves between the 3º and 5º nodes were selected and harvested for anatomic analysis from three individuals. Leaves used in histological sections were stored in pots with fixative solution ($FAA_{70\%}$) (Johansen, 1940). For dissociation of the leaf blade, the Jeffrey solution was used at a 10% concentration (Johansen, 1940), for a period of two days. After cleaning off the solution, sections were stored in 50% alcohol for 24 h and then stained in 1% safranin assembled in diluted glycerin.

Images using a scanning electron microscope (SEM) were obtained from 0.5 cm samples of species' leaves. First, leaf samples were dehydrated in an alcoholic series, followed by water withdrawal at the critical point, metallization and analysis in the SEM (Model: Zeiss Sigma-VP) (Silva, 2013). Botany terminology utilized for classification of secretory structures was done according to Fahn (1979) and Metcalfe & Chalk (1979).

Histochemical analysis was performed to locate oil secretory structures in sections by hand. Sudam III solution (Pearse, 1985) was used to detect lipid content, wherein a positive reaction is detected by an orange or bright-orange color. Images of histochemical reactions in secretory structures were obtained using a light microscope with an attached camera (Motic BA210).

2.3. Frequency of secretory structures

Secretory structures were quantified only in dissociated material which reacted positively in histochemical tests. For this, from each individual of *Croton sacaquinha* and *C. cajucara* (two morphotypes), fifteen leaves were dissociated. Leaf blades were sectioned in three regions: apex, median and base, and 30 fields of measurement were randomly selected. For petioles, 30 fields in three regions were evaluated. The collected data were inserted into the Balbach & Bliss (1991) formula to obtain mean values of structure per area.

$$\frac{\text{Nst}}{\text{Area}} = \frac{\text{X}}{1\ \text{mm}^2}$$

Where:
Nst: Number of secretory structures measured in each field of observation
Area: Area of objective of measurement
X: Number of secretory structures/1mm²

2.4. Extraction and essential oil yield

Essential oil yield of *Croton* leaves was conducted by extraction using the hydrodestillation technique using a Clevenger system. Leaves of three individuals of *C. sacaquinha* and *C. cajucara* morphotypes were harvested, weighed, fragmented, and inserted in a volumetric flask containing 500 mL of distilled water. Volumetric flasks were attached to the extractor system and attached to a heating plate (Santos et al., 2004).

Time of distillation was fixed at two hours, counted from the start of evaporation of water in the volumetric flask. After this period, the volume of oil was measured on the extractor system volumetric scale. Oil was removed and stored, adding 0.7 g of anhydrous sodium sulphate to complete absorption of moisture, and when two phases were formed, essential oil was separated and weighed (Girard et al., 2007, Vitti & Brito, 1999). For calculation of essential oil yield, the formula below was used:

$$\text{R\%} = \frac{\text{Voil x Doil}}{\text{Bm}} \text{ x } 100$$

Where:
R%: Essential oil yield in percentage value
Voil: Volume of essential oil (mL);
Doil: Essential oil density (g/mL);
Bm: Biomass (g).

2.5. Data analysis

Quantitative characteristics (number of structures and essential oil yield) were submitted to analysis of variance (ANOVA), followed by the Shapiro-Wilk normality test and mean comparison test (Tukey HSD) at a probability level α=0.05. A correlation analysis was also carried out using the software R (version 4.3.1) (R Core Team, 2023) and the packages: "corrplot" (Wei & Simko, 2021) "dplyr" (Wickham et al., 2023) and "ggplot2" (Wickham, 2016).

3. RESULTS

Croton sacaquinha and *C. cajucara* morphotypes presented trichomes in both the leaf blade epidermis and the petiole. In *C. sacaquinha* secretory trichomes spread throughout the epidermis, characterized by a main stem, peduncle-like and 1 or 2 central cells, in comparison to others, which are arranged horizontally and concrescent, being classified as porrect-stellate (Figures 2A and B). Unicellular glandular trichomes were seen in both morphotypes (Figure 2 C).

The morphotypes "common leaf" and "red leaf" of *C. cajucara* presented the same type of trichome: multiradiate stellate and glandular trichomes (Figures 2D - F). Stellate trichomes in this species are secretory and suspended by a stem composed of a cell column, being more frequent in leaf veins, mainly in those of first order. Unicellular glandular trichomes (Figure 2F) presented the same distribution. Petioles in *C. sacaquinha* and *C. cajucara* morphotypes presented the same trichome types found in their respective leaf blades.

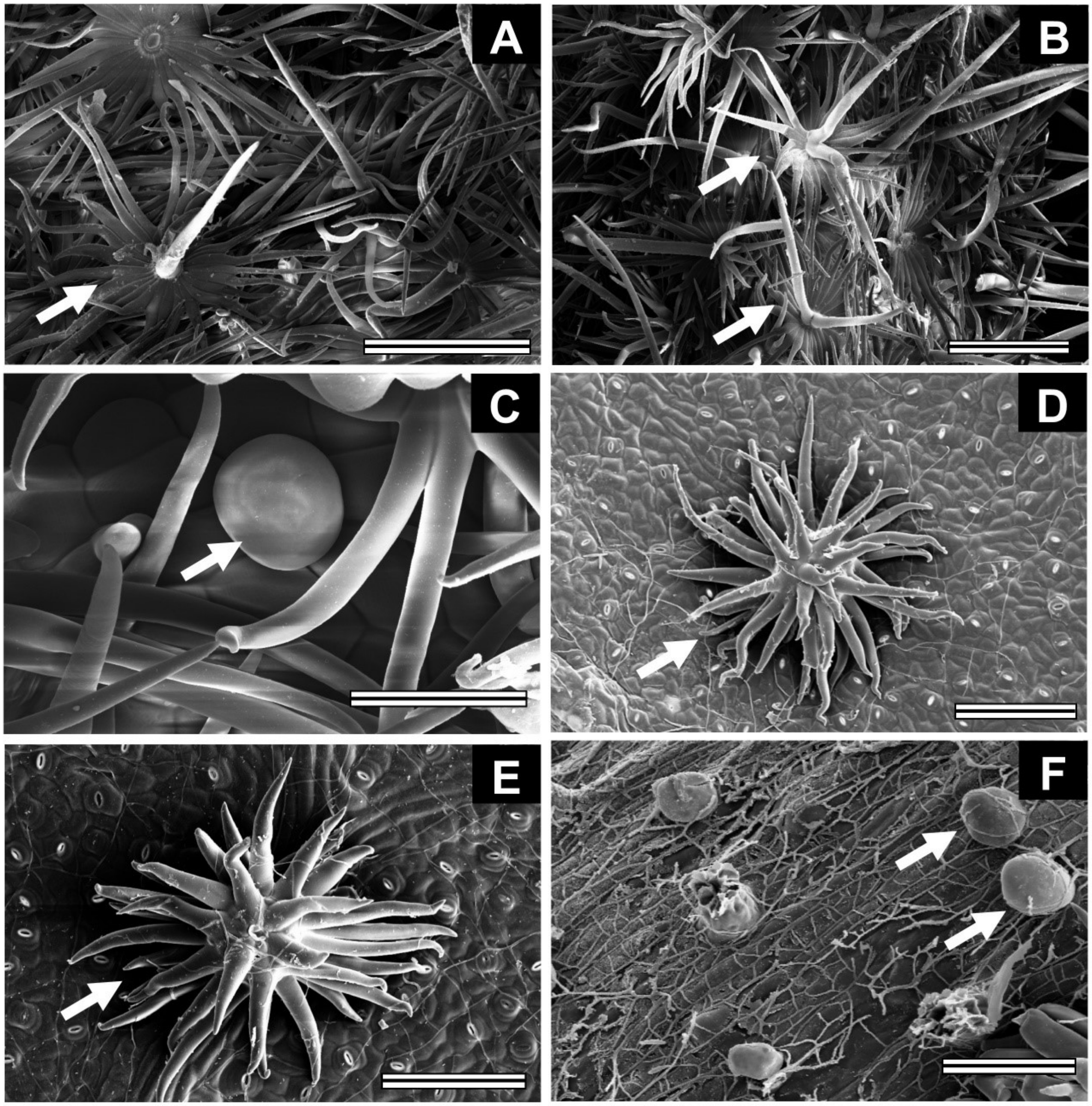

Figure 2. Secretory structures in *Croton sacaquinha Croizat*. In (A), (B) and (C), in *Croton cajucara Benth* "common leaf" morphotype (D) and (F) and in *C. cajucara* "red leaf" morphotype (E). Arrows: Stellate trichomes in (A), (B), (D) and (E) and glandular in (C) and (F). Scalebar: 20 µm in (C), 50 µm in (D), (E) and (F), and 100 µm in (A) and (B).

Histochemical tests applied to trichomes of leaves were positive for lipidic substances, being drop-shaped in *C. sacaquinha* in the inside and the base of porrect-stellate trichomes (Figure 3A), in idioblasts with a volatile nature which escape through epidermic cells, but with no obvious shape (Figure 3B), and in glandular trichomes as a spherical single content (Figure 3C).

In both morphotypes of *Croton cajucara*, the presence of lipidic substances was observed in glandular trichomes, as a spherical single content (Figure 3D) and like drops inside multiradiate stellate trichomes (Figure 3E).

With regard to quantification of secretory structures in leaf blades, statistical differences were observed only in the apex and median regions in *C. sacaquinha* and morphotypes of *C. cajucara* ($p<0.05$), and structures located in the leaf blade base differed solely between species (Table 1). In petiole regions, number of secretory structures was different comparing species (Table 1).

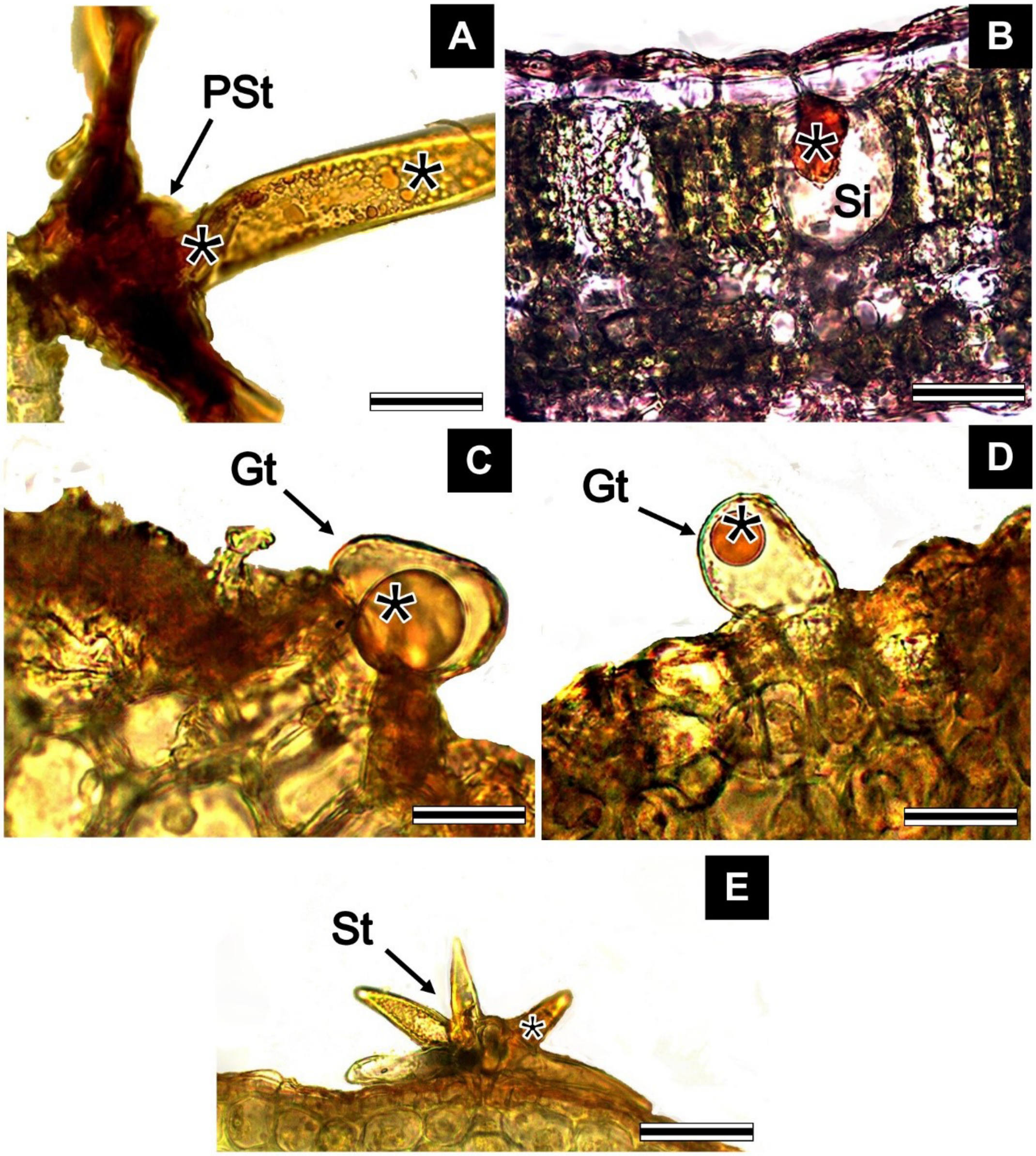

Figure 3. Histochemical test in secretory structures of leaves of *Croton sacaquinha* Croizat. (A), (B) and (C) and in *Croton cajucara* L. (D) and (E). Si: Secretory idioblast. St: Stellate trichome. PSt: Porrect-stellate trichome. Gt: Glandular trichome. Asterisk: lipophilic substance detection. Scalebar: 30 μm.

Table 1. Number of secretory structures of essential oil in *C. sacaquinha* Croizat. and two morphotypes of *C. cajucara* Benth, according to leaf regions.

Region		Species		
		C. cajucara **CL**	*C. cajucara* **RL**	*C. sacaquinha*
Leaf Blade	Apex	1.41 ± 0.28 c*	1.81 ± 0.33 b	4.13 ± 0.55 a
	Median	2.1 ± 0.37 b	1.59 ± 0.41 c	3.77 ± 0.54 a
	Base	2.04 ± 0.35 b	1.98 ± 0.5 b	3.55 ± 0.46 a
Petiole	Proximal	1.81 ± 0.28 b	1.84 ± 0.33 b	3.51 ± 0.55 a
	Medial	2.01 ± 0.37 b	1.91 ± 0.41 b	4.01 ± 0.54 a
	Distal	2.09±0.35 b	1.93 ± 0.5 b	5.06±0.46 a

*Mean values followed by standard deviation; means followed by different letters, in the rows, do not differ significantly (p≤0.05).

In general, *Croton sacaquinha* presented the highest values in the upper (3.56) and lower (7.87) epidermis, as well as the petiole (4.19) in comparison to *C. cajucara*. In turn, morphotypes did not differ between each other ($p \leq 0.05$) considering the total of secretory structures in both the epidermis and petiole (Figure 4).

Croton sacaquinha also presented the highest mean essential oil yield (0.63%). Morphotypes of *C. cajucara* did not differ in essential oil yield, varying from 0.34% (common leaf morphotype) to 0.36% (red leaf morphotype) (Figure 4). Essential oil yield was positively related with the number of secretory structures in both the epidermis and petiole (Figure 4).

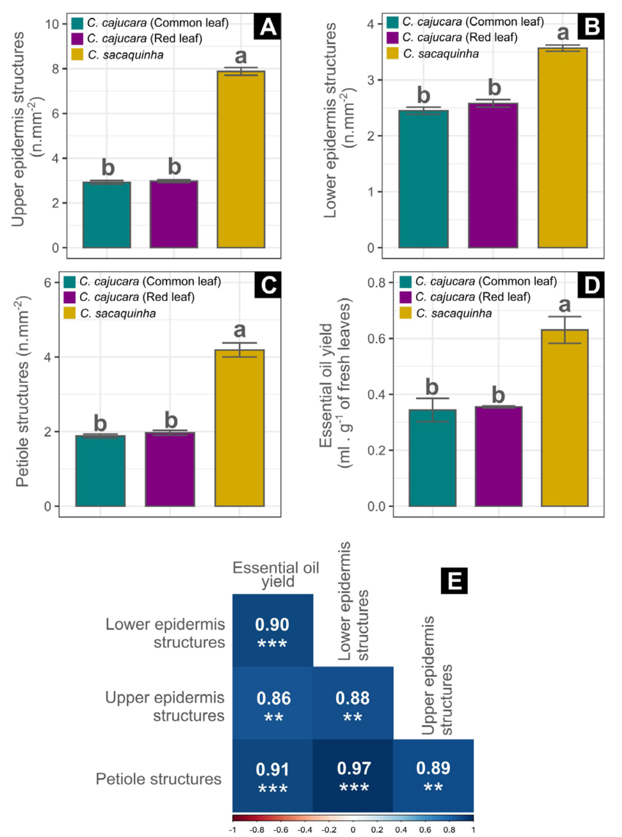

Figure 4. Quantitative analysis of *Croton cajucara* L. ("common leaf" and "red leaf" morphotypes) and *C. sacaquinha* Croizat. Secretory structures in upper epidermis (A), lower epidermis (B) and petiole (C). (D): Essential oil yield. (E): Correlation analysis between essential oil yield and number of leaf secretory structures. Different letters from (A) to (D) represent significant differences ($p<0.05$). In the correlation analysis: (**) $p < 0.01$ and (***) $p < 0.001$.

4. DISCUSSION

Trichomes in *Croton* vary in number and shape (Vitarelli et al., 2015), which was confirmed in this study by the observations of *C. cajucara* and *C. sacaquinha*. It is important to point out that the trichome terminology for species in *Croton* depends on the classification system employed (Metcalfe & Chalk, 1950, Webster et al., 1996). Stellate and lepidotes trichomes exhibit considerable morphological variation and are widely distributed across most *Croton* sections (Pinto-Silva et al., 2023). Furthermore, the porrect-stellate trichomes of *C. sacaquinha* closely resemble those observed in *C. spruceanus* (Pinto-Silva et al., 2023).

Secretory idioblasts were observed in *Croton cajucara* by Vitarelli et al. (2015), however this study did not report the presence of secretory trichomes in leaves of this species, which diverges from the results found here. Furthermore, unicellular glandular trichomes, as observed in both species and morphotypes analyzed in this study are also common to other species in *Croton*, such as *C. cordiifolius*, which occurs in the Northeast region of Brazil (Alves et al., 2017).

In relation to the number of secretory structures, *Croton sacaquinha* presented higher values than *C. cajucara* morphotypes, regardless leaf surface. As far as is known, this is the first time in which secretory structures in *Croton sacaquinha* and *C. cajucara* were quantified. Similar to *Croton cajucara*, in *C. amentiformis* leaves, the upper epidermis is almost glabrous, and trichomes are limited to the midrib; the lower epidermis, nonetheless, has the highest number of porrect-stellate trichomes, (Riina et al., 2015), which are also observed in *C. sacaquinha*.

Croton mollis Benth., another species that occurs in the Amazon region, presents a high number of stellate and secretory trichomes, which vary in length (Vitarelli et al., 2021). These structures, among others, were associated with adaptations to water balance, ensuring survival in flooded forest environments (Vitarelli et al., 2021). Although Feio et al. (2018) highlighted *Croton* trichomes as non-glandular, we emphasize the positive reaction in the histochemical tests, confirming the presence of lipophilic substances inside the trichomes in *C. cajucara* and *C. sacaquinha*.

The foliar structures evaluated here were positively related to essential oil yield of species. For this reason, *Croton sacaquinha* presented the highest extracted volume. Essential oil yield produced by *Croton* species can be influenced by many internal and external factors to the plant, and vary among the species (De Souza et al., 2017).

The composition of substances secreted by foliar structures are related to particular metabolic characteristics of each in individual. However, glandular secretory structures are known to produce and store a plant's essential oil (Biswas et al., 2009). A study done by Maurya et al. (2019) about the relationship between trichomes and essential oil content in species of *Ocimum*, showed the dependence of essential oil yield on number, size, and type of trichome.

Moreover, during the extraction process, some factors such as time of extraction have an influence on the obtained yield. Chaves et al. (2006) used the hydrodestillation technique in a Clevenger system for 4 hours and obtained 0.97% of essential oil yield with the *C. cajucara* "redleaf" morphotype. The employed technique to extract essential oils also can change the yield (Aziz et al., 2018). Mendonça et al. (2008), obtained 0.84 mL of yield for *C. cajucara* using steam-dragging extraction. Regarding the essential oil yield of other *Croton* species, Da Costa et al. (2022) reported a yield of 0.24% for *C. campinarensis* using the hydrodistillation method with a Clevenger apparatus over a 3-hour extraction period.

Overall, essential oils represent a diverse and complex source of terpenoids. The bitter taste of terpenoids in leaves (Yan et al., 2023) is a well-known deterrent to herbivory (War et al., 2018). The rupture of trichomes and the consequent release of terpenoid-rich volatile organic compounds (VOCs) may represent a defense strategy employed by *C. cajucara* and *C. sacaquinha* to safeguard photosynthetic tissues and increase survival rates. Furthermore, the emission of these VOCs may contribute to the defensive priming of *Croton* species neighboring plants (Heil & Bueno).

Many of these phytochemicals can be used by pharmaceutical, cosmetic and food industries. Similarly, as in other species, *Croton cajucara* and *C. sacaquinha* can be a source of bioactive compounds (Mendonça et al., 2008) and explored in a sustainable way, contributing to an increase in the income of Amazonian communities.

5. CONCLUSIONS

The anatomy of secretory structures and essential oil yield of both species of *Croton* (*C. sacaquinha* and *C. cajucara*), both of which occur in the Amazon region, were evaluated. *Croton sacaquinha* presented the highest values for foliar structures and essential oil yield. The results obtained provide benefits to taxonomy and biotechnology and highlight the potential of *Croton* species for sustainable use, extracting their essential oils as a non-woody forest product, which can contribute to an increase in the income of Amazonian communities. To advance the utilization of the essential oil of *Croton* species, future research efforts should focus on refining extraction methodologies and investigating potential applications, particularly in pharmacology and cosmetics. From an ecological perspective, investigations into the influence of volatile compound release on the stress responses of *C. cajucara* and *C. sacaquinha* are recommended.

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