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Effect of herbivory by goats on primary and secondary metabolism of *Cocos nucifera* L. (Arecaceae) in a semi-arid environment in Brazilian Northeast

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ABSTRACT

The relationship between herbivores and plants has important ecological implications for both organisms and directly affects the plant's physiological responses, which need to invest in structures and secondary metabolites to overcome the damages. This study aimed to evaluate functional attributes related to the primary and secondary metabolism of *Cocos nucifera* L. (Arecaceae) submitted to herbivory by goats. Five individuals of *C. nucifera* were selected in two areas, one with and one without goats. The carbohydrate content, specific leaf mass, and phytochemical screening were obtained, evaluating the presence or absence of saponins, tannins, flavonoids, steroids, alkaloids, and glycosides. The results show that plants under herbivory showed 50% more sugars and a higher specific leaf mass than no-herbivory plants. Also, plants under herbivory showed a higher amount of saponins, steroids, and alkaloids. The production of defense metabolites, which are energetically costly, require the energy supply provided by the carbohydrates produced in photosynthesis; thus, higher levels of sugars were observed in attacked plants. Even under attack, *C. nucifera* plants allocate resources for biomass production, to increase leaf sclerophylly and hinder herbivory. The investment in saponins, steroids, and alkaloids is related to herbivory, as demonstrated by the analysis of principal components analysis. We conclude that herbivory by goats activates defense responses in *C. nucifera* plants, making them allocate sugars to produce secondary metabolites.

Keywords: Biotic stress, plant physiology, photosynthesis, saponins, alkaloids, steroids.

RESUMO

A relação entre herbívoros e plantas tem implicações ecológicas importantes para ambos os organismos, e afeta diretamente as respostas fisiológicas das plantas, que necessitam investir em estruturas e metabólitos secundários a fim de combater os danos causados. Esse estudo objetivou avaliar atributos funcionais do metabolismo primário e secundário de *Cocos nucifera* L. (Arecaceae) submetidas a herbivoria por caprinos. Cinco indivíduos de *C. nucifera* foram selecionados em duas áreas, uma com e outra sem caprinos. Foram avaliados o teor de carboidratos, a massa foliar específica, e triagem fitoquímica, avaliando a presença ou ausência de saponinas, taninos, flavonóides, esteróides, alcalóides e glicosídeos. Os resultados mostram que as plantas herbivoradas apresentaram 50% mais açúcares, e maior massa foliar específica significativamente maior que as plantas não herbivoradas. Além disso, as plantas herbivoradas apresentaram maior quantidade de saponinas, esteróides e alcalóides. A produção de metabólitos de defesa, custosos energeticamente, necessitam do aporte de energia fornecido pela quebra dos carboidratos produzidos na fotossíntese; assim, maiores teores de açúcares foram observados nas plantas herbivoradas. Mesmo sob ataque, as plantas de *C. nucifera* alocam recursos para produção de biomassa, a fim de aumentar a esclerofilia das folhas e dificultar a herbivoria. O investimento em saponinas, esteróides e alcalóides está diretamente relacionado à herbivoria, como demonstra a análise de componentes principais.

Concluimos que a herbivoria por caprinos ativa respostas de defesa nas plantas de *C. nucifera*, fazendo com que elas aloquem açúcares para a produção de metabólitos secundários.

Palavras-chave: Estresse biótico, fisiologia vegetal, fotossíntese, saponinas, alcalóides, esteróides.

Introduction

The herbivore-plant relationship is essential in the distribution and abundance of species across time and space, in the adaptations responsible for diversity, and the implications for ecology and evolutionary processes (Corrêa et al., 2008). Herbivory leads to tissue loss of the plant, be resulting in changes in its physiology, modifying its metabolism, and compromising aspects such as growth, reproduction, and survival of plant species (Dourado et al., 2016). Velasque & Del-Claro (2016) state that the impact of herbivory on plant performance is dependent on its phenology (stage of development), the part consumed by the animal, and the intensity and frequency of the attack. According to Leal, Vicente & Tabarelli (2016), wild and domesticated goats have been recognized as significant sources of vegetation degradation in arid environments around the world, causing damage to the distribution and growth of a various tree, shrub, and herbaceous species.

The practice of goat farming in the Brazilian Northeast is responsible for a large part of economic activity, with its products used for personal and commercial use (Paulo & Lopes, 2014). Domestic goats are small ruminants, capable of consuming all parts of the plant, from the roots to the leaves. Also, they are animals fully adapted to semi-arid conditions due to their digestive efficiency and capacity to save water and nitrogen, which is essential to withstand the dry months (Medeiros, 2000).

To cope with herbivory pressures, plants have developed mechanical and chemical defense systems against herbivores (War et al., 2018). Mechanical defenses involve the production of trichomes, sclerophylly, latex production, among others, and chemical defenses are related to the production of primary and secondary metabolites with anti-herbivory effect (Kaur, Gupta & Taggar, 2015; Erb, 2018). Thus, plants can face herbivory both by hindering the herbivore's access to consumable parts of the plant and by affecting the digestibility of the material consumed (Tomlinson et al., 2016). The way the plant allocates resources for both types of defense varies in time and space and is directly related to the type of damage caused, the organ affected by herbivory, and the specificities of the plant's life history (Züst, Rasmann & Agrawal, 2015; Xiao et al., 2019).

The herbivore cannot consume all the edible parts of the plant. During the attack, the plant develops systemic responses that signal to produce defense compounds in parts not yet consumed in the plant, to avoid further damage (Vos et al., 2013). This type of systemic defense involves the production of hormonal signalers such as jasmonic acid and salicylic acid (Soler, Erb & Kaplan, 2013). Also, herbivory affects CO₂ assimilation and, consequently, the allocation of biomass for growth, since much of the energy investment is directed towards the production of defense metabolites, which are costly in terms of energy (Peschiutta et al., 2018).

The Brazilian Northeast region comprises the largest population of small ruminants in Brazil, with more than seven million goats and nine million sheep that constitute an important economic activity for the population (IBGE, 2017). Besides, the region has about 90% of the coconut palm *Cocos nucifera* L. (Arecaceae), as its growth is favored in regions with a semi-arid climate, marked by high temperatures and periods of drought (Azevedo et al., 2006; Câmara et al., 2018). Thus, in the driest periods of the year, the coconut tree is one of the only sources of biomass available to goats and other herbivores. Thus, the objective of this work was to evaluate the changes in morphological, biochemical, and phytochemical attributes in plants of *C. nucifera*, submitted to herbivory pressure by goats. Our central hypothesis is that plants under herbivory have a higher leaf content of carbohydrates, a greater specific leaf area, and investment in secondary metabolites when compared to non-herbivore plants.

Materials and Methods

The experiment was carried out in the municipality of Calçado, located 29 km from Garanhuns, Pernambuco, Brazil (8°44'31" S, 36°20'02" W). The site has an average annual temperature of 24°C, and average precipitation of 765 mm (Figure 1), with a risk of drought greater than 60%, which qualifies it as belonging to the semi-arid climate (Min, 2005). The data collection was performed in August 2019. Two areas were selected for the study, one fenced and protected from the interference of goats, and the other free from fences, both containing at least five mature individuals of *C. nucifera*. To determine that the functional variations observed in the plants were

caused by herbivory, and not by differences in soil water content, we carried out the analysis of soil moisture. Five soil samples were collected, packed in ziplock bags to avoid loss of moisture, and taken to the laboratory to obtain the wet weight. The samples were submitted to a forced ventilation oven at 60°C for 72 hours to obtain dry weight. The soil moisture was obtained by the difference between wet weight and dry weight. From the results, there was no evidence of soil patches with significantly different water content. Ten leaves were collected from five individuals, in areas with or without goats, to carry out the phytochemical analysis. All individuals were between 1.5 m and 2 m tall, with leaves at height available to goats. The leaves of the herbivory treatment were selected from the observed bite pattern (González-Pech, Torres-Acosta & Sandoval-Castro, 2014). The herbivory rate was calculated by scanning the herbivorous leaves and digitally complementing the removed area, following the methodology described by Rasband (1997).

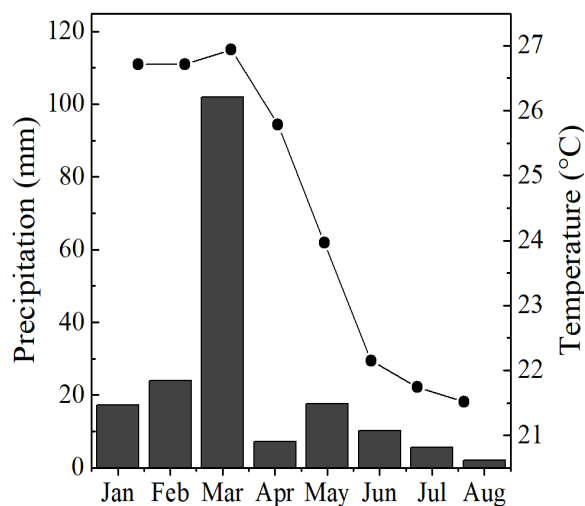


Figure 1. Precipitation and the average temperature of the first eight months of 2019, in the Calçado municipality, Pernambuco, Brazil. Font: Silva et al. (2020).

To assess biomass production and the allocation of biomass in plants, we performed analyzes of total soluble sugars and specific leaf mass. Five leaves of each treatment (herbivory and no-herbivory) were collected and immediately taken to a freezer at -20°C. The determination of total soluble sugars was based on a colorimetric analysis in a spectrophotometer calibrated at 490 nm, according to Dubois (1956). The specific leaf mass was obtained using five leaves of each treatment, packed in paper bags, and taken to the laboratory for processing. Each leaf was scanned on a flatbed scanner to calculate the leaf area, using the ImageJ software. Subsequently, the leaves were

taken to a forced ventilation oven at 60°C for 72 hours to obtain the dry weight. The specific leaf mass was calculated by the ratio between dry mass and leaf area, according to the protocol described in Pérez-Harguindeguy et al. (2013).

To evaluate the phytochemical composition of *C. nucifera* herbivory and no-herbivory treatments, a qualitative analysis of secondary metabolites was performed. Five leaves from each treatment were collected, taken to the laboratory, dried in a forced ventilation oven at 60°C for 72 hours, and ground to obtain a fine powder. Then, 500 mg of each sample was immersed in 30% ethanol and taken to decoction for 10 minutes in a water bath. Subsequently, the samples were filtered on filter paper, and the ethanolic extract used for phytochemical screening. Saponins, tannins, flavonoids, alkaloids, steroids, and glycosides were analyzed. The results were expressed in a table of presence (+) and absence (-), assigning a broader classification according to the strength of the sample's color: presence (+); presence with stronger coloring (++); presence with a much stronger color (+++), showing the presence in greater amounts of the analyzed metabolite. The secondary metabolites analysis was performed as follow:

Saponins: 2 mL of extract + 2 mL of chloroform + 5 mL of water in a test tube, shaking vigorously for 3 minutes. The presence of foam indicates the presence of saponins (Silva, Miranda & Conceição, 2010).

Tannins: 2 mL of extract + 3 drops of 1% ferric chloride (alcoholic), stirring vigorously. Blue color indicates the presence of water-soluble tannins, and green color indicates the presence of condensed tannins (Silva, Miranda &

Flavonoids: 2 mL of extract + 1.5 cm of magnesium on tape + 1 mL of hydrochloric acid. The reddish-pink color indicates the presence of flavanols, violet the presence of flavones, and orange, the presence of Conceição, 2010). flavones (Mouco, Bernardino & Cornélio, 2003). The absence of color indicates the presence of isoflavones and chalcones (Oliveira, Akisue & Akisue, 1998).

Alkaloids: 2 ml of the extract + 15 drops of 1% sodium hydroxide + 2 ml of water + 2 ml of chloroform. The aqueous fraction was discarded, and 15 drops of 1% HCL and 2 ml of water were added. Subsequently, the chloroform fraction was discarded, and three drops of the Dragendorff reagent were added to the medium. Orange coloring indicates the presence of alkaloids (Silva, Miranda & Conceição, 2010).

Steroids: 1 mL of extract + 3 mL of chloroform + 2 mL of acetic anhydride + 1 mL of

sulfuric acid. The formation of a blue-green ring indicates the presence of steroids (Goyal, Middha & Sen, 2010)

Glycosides: 2 mL of the extract + 1 mL of acetic acid + 1 mL of 5% iron chloride + 1 mL of sulfuric acid. The bluish-green color indicates the presence of glycosides. (Goyal, Middha & Sen, 2010).

Data were tested for normality and homogeneity. Considering to compare total soluble sugars and specific leaf mass between treatments, a T-test at 5% probability was performed. Pearson's correlation analyzes were also performed between the herbivore rate and total soluble carbohydrates and the specific leaf mass. All analyzes were performed using the Statistica 8.0 software. The data of presence and absence of metabolites were transformed and assigned weights ranging from 1 (in the absence) to 3 in the presence, as a stronger color, than the average. Then, the data were submitted to a Principal Component Analysis (PCA) to determine whether the analyzed leaf attributes would be able to separate the two groups (herbivory and no-herbivory) spatially and to identify which leaf attributes would be the main responsible for the separation between the treatments. The PCA was performed using the software Origin 8.0 (Origin Lab).

Results and Discussion

After analyzing the soil moisture, it was observed that the average humidity of the experimental area was 4.3%, not showing soil patches with differentiated humidity. The temperature did not exceed 27°C, which is ideal for the development of the coconut tree (Câmara et al., 2018), ensuring that environmental variations did not cause the changes found in the plants, since unfavorable environmental conditions such as relevant variations in precipitation amount or air temperature cause morpho-physiological changes in plants (Verma, Nizam & Verma, 2013).

The plants of the herbivory treatment had a total soluble sugars content 50% higher than the plants of the no-herbivory treatment ($p < 0.05$) (Figure 2A). When subjected to herbivory, plants start the production of defense metabolites to minimize the damage caused by the herbivore and signal other parts of the plant about the attack (Ferrieri et al., 2015). These metabolites need energy to be produced, and this energy is provided by the breakdown of sugars generated in the photosynthetic process (Machado, Baldwin & Erb, 2017). Thus, herbivory treatment plants are subject to a trade-off between defense and growth needs; that is, they need to modulate the allocation of resources for the production of defense

metabolites, which are very costly in terms of energy, instead of the healthy development of the plant (Zhou et al., 2015).

Interestingly, if we analyze only the data of specific leaf mass, this trade-off between defense and growth does not seem to be accurate, since herbivory treatment plants showed significantly higher specific leaf mass (1.51 g.cm^{-2}) than plants of the no-herbivory treatment (1.02 g.cm^{-2}) (Figure 2B). In other words, they allocate a significant number of resources to the production of biomass even under the attack of goats. However, it must be remembered that defenses can be both morphological and biochemical (Belete, 2018). The increase in specific leaf mass in low nutrient environments, such as semi-arid, is associated with increased sclerophylly, an important morphological characteristic in defense against herbivory, since thicker and harder leaves hinder the mechanical damage and digestibility of herbivores (Neves et al., 2009). The correlation data between soluble sugars and specific leaf mass, and the herbivory rate (Figure 2CD) corroborate that changes in both primary metabolism and leaf morphology are strongly associated with herbivory rate (0.81 and 0.93, respectively).

It is possible to verify differences in secondary metabolite's investment between the two treatments in this study (Table 1). The only class of secondary compounds that was observed only in herbivory treatment was steroids. However, attacked plants showed a higher amount of saponins and alkaloids than no-herbivory treatment plants. Steroids are important components of membranes and are precursors of plant hormones related to defense responses against biotic stress, such as brassinosteroids (Bajguz & Hayat, 2009; Behmer, Grebenok & Douglas, 2011). This class of phyto regulators acts to increase tolerance to biotic stress through the activation of enzymes, resistance genes, and transcription factors to reduce the damage caused by the attack (Anwar et al., 2018).

Saponins influence various biotic relationships in plants, from responses to attack by herbivores and pathogens to symbiosis with mycorrhizal fungi (Mugford & Osbourn, 2012). The saponin molecule is composed of a hydrophobic portion, usually a triterpene or sterol, and a portion formed by a hydrophilic carbohydrate chain (Hussein et al., 2019). This amphipathic characteristic of the saponin molecule confers its biological activity. Among other effects, saponins hinder protein digestion through the inhibition of proteinases and cause enterotoxicity due to their surfactant characteristics (Soetan, Ajibade &

Akinrinde, 2014). Alkaloids, as well as saponins, found in higher quantities in plants under herbivory, have a recognized effect in the case of biotic stress. This class of nitrogenous metabolites does not have a recognized fundamental action in plant physiology. However, they act in defense against herbivores, affecting various metabolic systems, such as the production and activation of enzymes, DNA synthesis, and affecting the nervous system of animals in some cases (Wink, Schmeler & Latz-Bruning, 1998; Mithöfer & Boland, 2012).

Equally interesting is to analyze the metabolites that are in greater quantity in no-herbivory than in herbivory treatment plants, such as condensed tannins. Condensed tannins are a

class of phenolic compounds with a recognized role in defense against herbivory, due to their ability to precipitate protein, affecting digestibility (Constabel, Yoshida & Walker, 2014). In a study evaluating the effect of insect defoliation on *Populus tremuloides*, Madritch & Lindroth (2015) found that the content of condensed tannins is correlated with the recomposition of nitrogen content in the plant after a considerable loss of biomass. Thus, it would be more advantageous for the plant to maintain lower levels of condensed tannins as a form of protection against herbivory. In other words, keeping small amounts of condensed tannins would affect the nitrogen precipitate protein, affecting digestibility (Constabel, Yoshida & Walker, 2014).

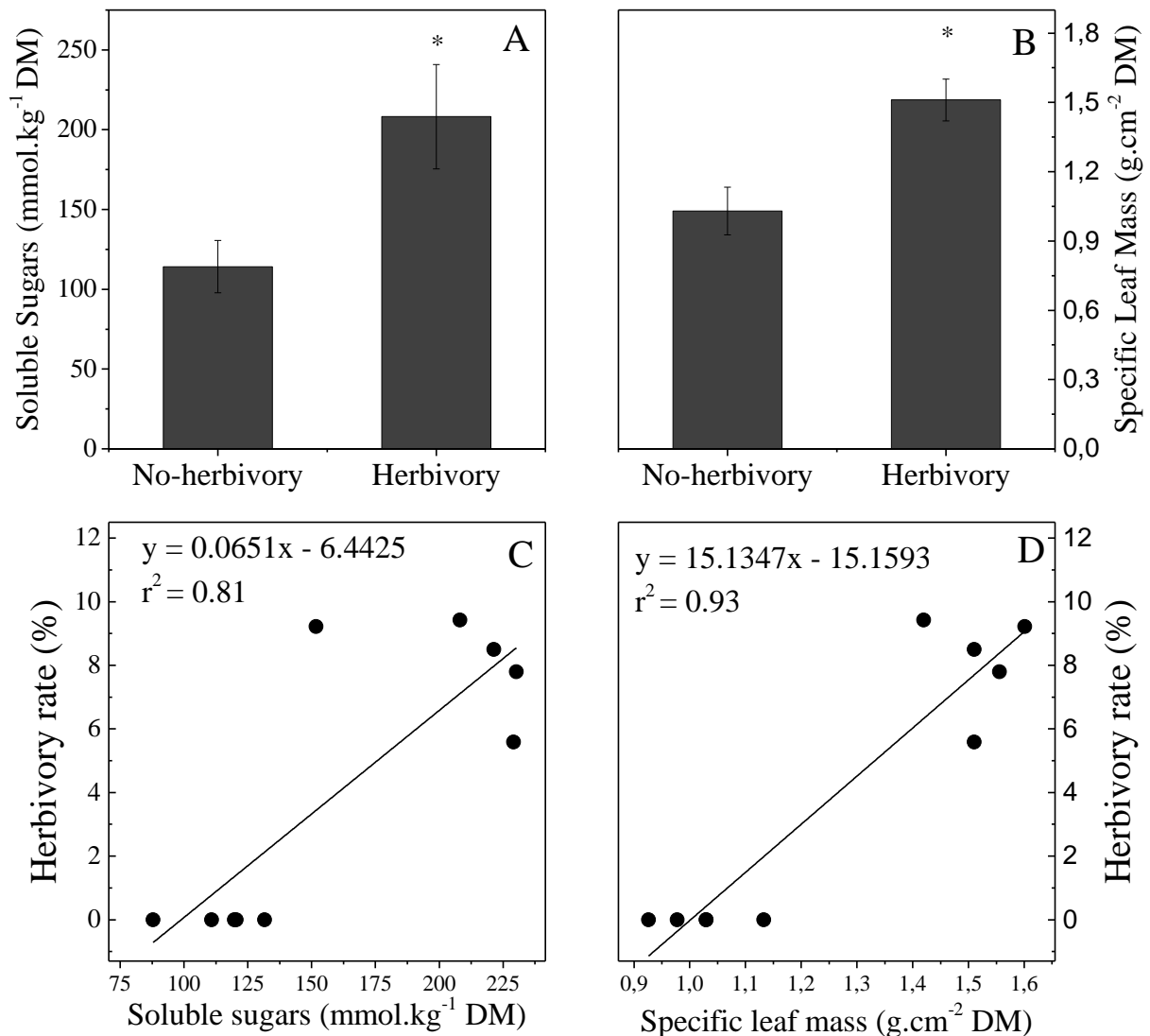


Figure 2. Total soluble sugars content (A); specific leaf mass (B); and relations between herbivory rate, soluble sugars content, and specific leaf mass (C-D) in *Cocos nucifera* L. (Arecaceae) leaves subjected to herbivory pressure by goats, in Calçado municipality, Pernambuco, Brazil. Bars \pm standard deviation with an asterisk (*) differed significantly from the non-herbivore control treatment ($p < 0.05$), DM = Dry Matter. $n = 5$. Font: Silva et al. (2020).

Table 1. Secondary metabolites in leaves of *Cocos nucifera* L. (Arecaceae) subjected to herbivory pressure by goats in Calçado municipality, Pernambuco, Brazil. (-) absence; (+) presence; (++) presence in high quantity; (+++) presence in greater quantity; n = 5. Font: Silva et al. (2020).

Metabolites	No-Herbivory	Herbivory
Saponins	+	++
Hydrolyzed Tannins	-	-
Condensed Tannins	++	+
Flavonoids (flavones)	+	+
Alkaloids	+	++
Steroids	-	+
Glycosides	+	+

An overview of all the data and how much they influence the differentiation between herbivory and no-herbivory treatments can be seen through the principal component analysis (Figure 3). The analyzed variables were responsible for explaining 83.3% of the data variation (PC1 - 70%; PC2 - 13.3%). It is clear that the separation of the groups mostly followed the variation of the data on

the PC1 axis. The herbivory treatment plants showed higher correlation values with soluble sugars (0.98), saponins (0.98), alkaloids (0.96), specific leaf mass (0.80), and condensed tannins (-0.95). These results demonstrate the direct and powerful relationship between the herbivory caused by goats and the plant's investment in these leaf attributes.

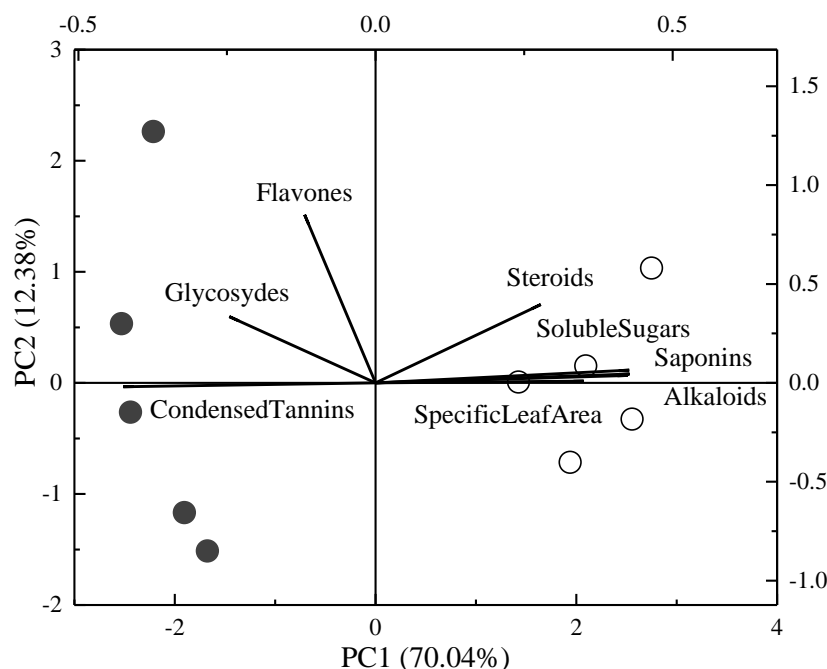


Figure 3. Principal component analysis of the data collected from *Cocos nucifera* L. (Arecaceae) submitted to herbivory pressure by goats in Calçado municipality, Pernambuco, Brazil. Font: Silva et al. (2020).

Conclusion

The herbivory caused by goats in *Cocos nucifera* L. promotes the plant's investment in sugars, specific leaf mass, and secondary metabolites such as saponins, alkaloids, and steroids, corroborating our central hypothesis. Other classes of compounds, such as condensed tannins, can indirectly affect herbivory rates by modulating other physiological processes of the plant, such as nitrogen recovery after an attack. Thus, it was concluded that both biochemical and

morphological changes can be attributed to herbivory by goats, as demonstrated by the analysis of principal components since there was no variation in water availability in the soil, and the plants were submitted to the same atmospheric conditions. These results highlight the influence of biotic stress on the ecophysiological responses of a plant species of great economic importance for the Brazilian Northeast.

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