

Communication

Is the Mycorrhizal Activity Related to the Accumulation of Foliar Bioactive Compounds in *Anadenanthera colubrina*?

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Abstract

Arbuscular mycorrhizal fungi positively modulate the production of plant bioactive compounds under greenhouse conditions and in experimental fields; however, their role in naturally occurring trees is poorly understood. This research aimed to verify the relationship between mycorrhizal activity and the concentration of foliar biomolecules in a natural population of *Anadenanthera colubrina*. Leaves and rhizosphere soil were collected from ten individuals of two fragments during the dry and rainy seasons. Foliar metabolite concentrations were quantified, while the soil was used to evaluate mycorrhizal colonization and glomalin-related soil proteins (GRSPs). Mycorrhizal colonization showed a positive correlation ($p \leq 0.05$) with metabolite production only during the dry season. GRSPs has higher deposition during the dry season, but has a positive correlation ($p \leq 0.05$) with metabolite production only in the rainy season. Both seasons influenced metabolite concentration, with the highest accumulation occurring during the rainy season. Therefore, mycorrhizal colonization and GRSPs deposition are related to the production of foliar metabolites of *A. colubrina* in a natural environment, but this relationship is strongly modulated by local rainfall.

Keywords: arbuscular mycorrhizal fungi; Fabaceae; metabolites; rhizosphere



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1. Introduction

It is known that arbuscular mycorrhizal fungi (AMF) can enhance the production of bioactive compounds from plants grown in greenhouse [1,2] and field conditions [3,4]. The mycorrhizal benefits have been unraveled in various herbs and tree species [5–8]; nevertheless, it remains unclear whether mycorrhizal activity is associated with metabolite accumulation in naturally occurring forest plants.

Anadenanthera colubrina (Vell.) Brenan is a suitable species for investigating this relationship. This deciduous species possesses therapeutic potential [9–12] due to the accumulation of bioactive compounds such as flavonoids in the phytomass [13–16]. In this context, some

factors are known to influence the production of these bioactive metabolites in *A. colubrina*, including AMF [17]. However, the mycorrhizal benefits were observed on seedlings grown under controlled conditions [17–21], with no reports on the relationship between these microorganisms and the production of bioactive compounds under natural conditions.

Furthermore, other factors that may modulate the production of plant bioactive compounds are seasonality and rainfall [22–24]. Such effects on metabolite production in *A. colubrina* have been reported [25,26]; however, these studies focused primarily on phenolic compounds and did not consider the activity of rhizosphere microorganisms, such as AMF, especially in relation to the biosynthesis of bioactive compounds in leaves, despite this organ being a renewable resource [27] that can be used by the industry of phytoformulations, instead of stems [13,16].

Therefore, the hypothesis that mycorrhizal activity is related to the production of foliar bioactive metabolites in a natural population of *A. colubrina* was tested. This study aimed to evaluate whether there is a correlation between mycorrhizal activity and the production of foliar bioactive compounds in a natural *A. colubrina* population.

2. Materials and Methods

The plant material and rhizosphere soil were collected from a natural *A. colubrina* population in two fragments of a Seasonal Atlantic Forest near the road in the municipality of Timbaúba, Pernambuco, Brazil (7°31'52.1" S; 35°19'09.9" W). Climatic conditions are described in Table 1. Samples were collected from two fragments to ensure greater representativeness of the population. The plots had a total area of approximately 4000 m² (fragment 1) and 8000 m² (fragment 2), with a total distance of 4000 m between the fragments (Figure 1). Biochemical, phytochemical, and mycorrhizal parameter analyses were performed at the Laboratory for Analysis, Research, and Studies in Mycorrhizae, University of Pernambuco (LAPEM/UPE), Brazil (8°2'47.143" S; 34°53'15.086" W).

Table 1. Weather conditions of the sampling points (Timbaúba, Pernambuco, Brazil) from a natural *Anadenanthera colubrina* (Vell.) Brenan population.

Attribute	Characteristic
Climate	Tropical with precipitation during fall–winter
Classification (Köppen)	As (tropical hot and humid climate)
Raining Season	March–July
Dry Season	August–February
Potential evapotranspiration	1450–1550 mm/year
Approximate relative humidity	100%
Temperature (October 2024)	23–3 °C
Temperature (March 2025)	24–33 °C
Accumulated precipitation (October 2024)	9.2 mm
Accumulated precipitation (March 2025)	178.5 mm

Sources: APAC [28–30]; Koppen Brasil [31]; WeatherSpark [32].

The experimental design was completely randomized, with two treatments: (1) leaves and rhizosphere soil collected during the dry season, and (2) leaves and rhizosphere soil collected during the rainy season, in ten replicates. Each replicate consisted of three subsamples of leaves or soil.

For each of the ten *A. colubrina* individuals, three leaf samples were collected from the lower branches and at the edges of the canopy, at equidistant points, forming a composite sample for each individual. A composite sample of rhizosphere soil was also collected at three equidistant points in the canopy projection, at up to 20 cm depth (Figure 2). Sampling was conducted in October 2024 (dry season, during leaf fall) and March 2025

(rainy season, with young to mature leaves). The individuals were spaced at least 12 m apart and had their trunks marked with metal tags for identification. Plant material was also collected for the preparation of herbarium specimens and species confirmation, which were deposited in the Geraldo Mariz Herbarium—HUFP (Voucher: 101925), at the Federal University of Pernambuco (UFPE), Brazil ($8^{\circ}03'02.4''$ S $34^{\circ}56'52.0''$ W). All sample collection procedures (vegetation and rhizosphere) were registered in *SisGen* (*Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado*) (ACBD963).

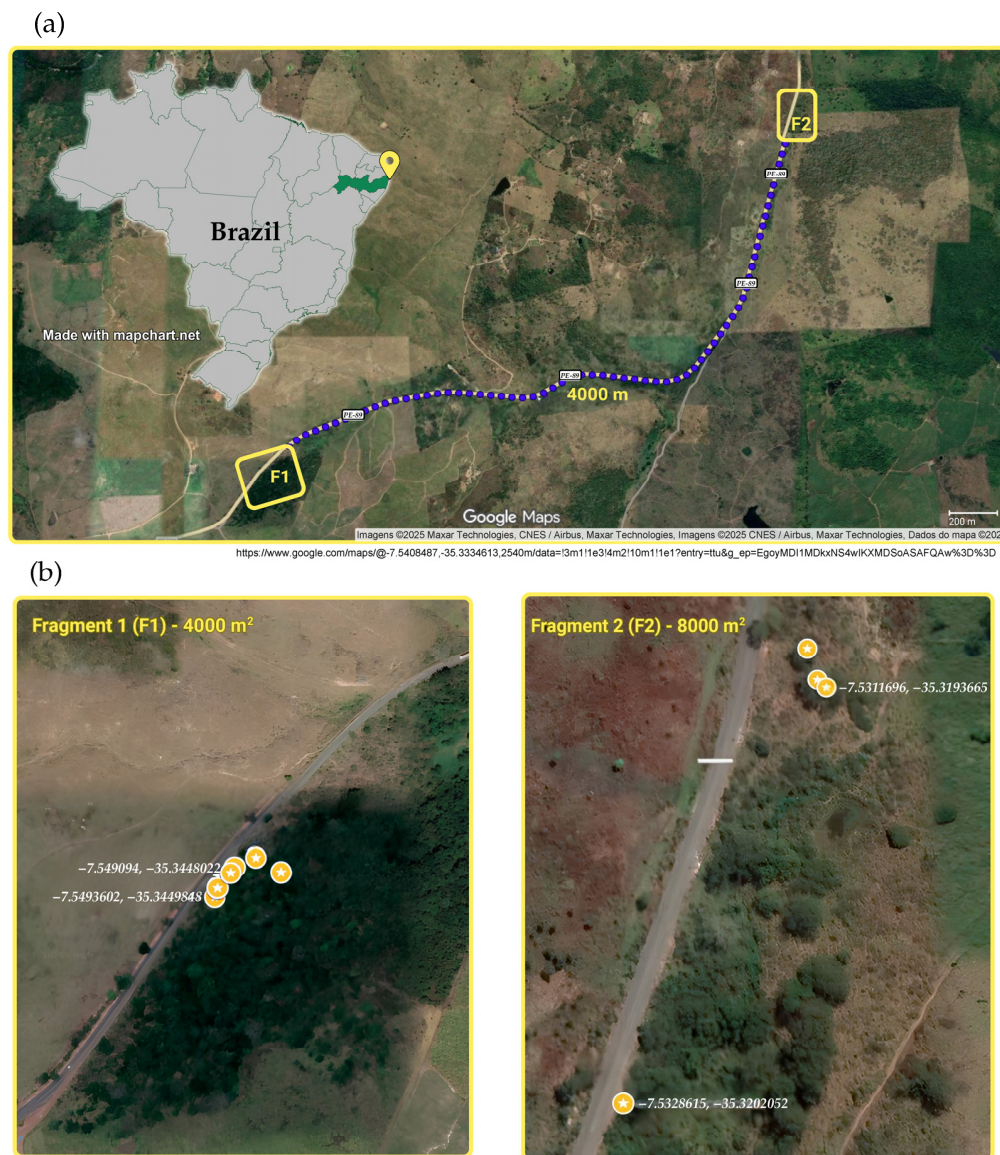


Figure 1. Brazilian map highlighting the state of Pernambuco with the sampling area (a), and details of the two fragments (b), and sampling points (stars) on the road in the municipality of Timbaúba (PE-89, Pernambuco, Brazil) ($7^{\circ}31'52.1''$ S; $35^{\circ}19'09.9''$ W). Image created using mapchart.net, Canva.com, and maps.google.com.

From the soil samples collected each season, two composite samples were prepared and characterized by the Agronomic Institute of Pernambuco (IPA) (Table 2). The soil was air-dried at room temperature, and the roots were retrieved for rhizosphere analysis. The leaves were superficially cleaned, thermally stabilized (72 h, 45°C), chopped, and aliquoted (500 mg). The aliquots were transferred to amber flasks containing 20 mL of ethanol (95%) (Química Moderna[®], São Paulo, Brazil), and ultrasonic-assisted extraction was carried out (100 W, 40 kHz, 30 min) (SolidSteel[®], São Paulo, Brazil) [33] (adapted). The extract was

filtered through qualitative filter paper (Celab[®], Recife, Pernambuco, Brazil) and stored ($-18\text{ }^{\circ}\text{C}$) until analysis. The concentrations of total proteins (TPtC), total phenols (TPC), total tannins (TTC), total proanthocyanidins (TPcC), total flavonols (TFoC), total flavonoids (TFC), and total saponins (TSC) were evaluated in this extract (Figure 2).

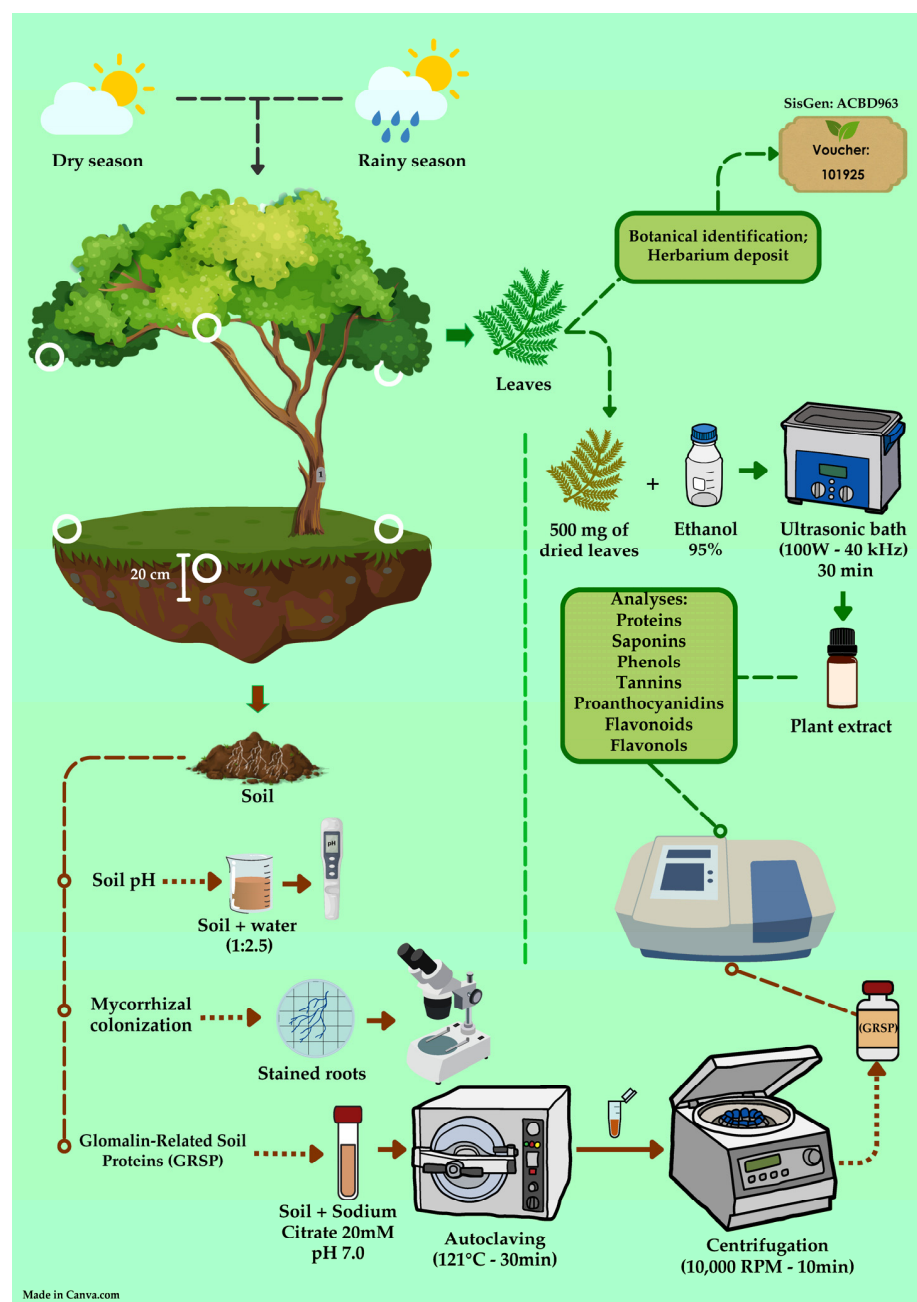


Figure 2. Summary of the sampling methodology, foliar biomolecule evaluations, and mycorrhizal activity. SisGen: Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado; GRSP: glomalin-related soil proteins. Image created in Canva.com.

To quantify TPtC, the Bradford method in [34] (adapted) was used, with bovine serum albumin (BSA) (Neon[®], São Paulo, Brazil) as the standard curve ($y = 0.0007x - 0.0063$, $R^2 = 0.9997$). TPC was quantified by the Folin–Ciocalteu reduction method (Merck[®], Darmstadt, Germany) [35] (adapted), using gallic acid (Dinâmica[®], São Paulo, Brazil) to generate the standard curve ($y = 0.0071x + 0.0352$, $R^2 = 0.9949$). Additionally, TTC was estimated by the casein complexation method (Vetec[®], Rio de Janeiro, Brazil) [25] (adapted), considering the formula: [Total tannins] = [Total phenols] – [Remaining phenols].

Table 2. Characterization of soil collected from the rhizosphere of *Anadenanthera colubrina* (Vell.) Brenan during the dry and rainy seasons in fragments of the seasonal Atlantic Forest in Timbaúba, Pernambuco, Brazil (7°31'52.1" S; 35°19'09.9" W).

Sampling Period	P	Ca	Mg	Al	K	H	S	CEC	Density	Soil Particle Size Distribution	Residual Moisture	Available Water
	(mg dm ⁻³)				(μmolc dm ⁻³)				(%)	Cs:Fs:S:Cl * (%)	(%)	(%)
Dry season	37	8.70	4.30	0.00	0.68	5.77	13.8	19.6	2.45	29:26:25:20	3.25	14.57
Rainy season	33	8.25	4.50	0.00	0.52	5.19	13.4	18.6	2.45	26:28:26:20	3.25	14.76

* Cs—Coarse sand; Fs—Fine sand; S—Silt; Cl—Clay. CEC = Cation Exchange Capacity.

The TPcC was measured using the interaction method with acid vanillin (2%, Química Moderna®, São Paulo, Brazil) [36] (adapted), using catechin (Sigma-Aldrich®, São Paulo, Brazil) as the standard curve ($y = 0.0412x + 0.0026$, $R^2 = 0.9999$). For the determination of total TFC, the complexation method with Aluminum chloride (Dinâmica®, São Paulo, Brazil) [37] (modified) and rutin (Sigma-Aldrich®, São Paulo, Brazil) was adopted as the standard ($y = 0.0024x - 0.085$, $R^2 = 0.9971$). The TFC was determined using the method described by Woisky and Salatino [38] and Bencherif et al. [39] (adapted), with quercetin used to generate the standard curve ($y = 0.0063x - 0.0231$; $R^2 = 0.9982$). TSC was quantified by the cobalt chloride complexation method (Nuclear®, São Paulo, Brazil) [40], using saponin (Alamar Tecno Científica Ltda., São Paulo, Brazil) for the standard curve ($y = 0.0009x + 0.0045$, $R^2 = 0.9926$).

For rhizosphere evaluations, soil pH, mycorrhizal colonization, and glomalin-related soil proteins (GRSPs) production were determined (Figure 2). Soil pH in H₂O was evaluated according to Silva [41] and Tiquia [42] (modified). For mycorrhizal colonization, the roots were washed, clarified, acidified, and stained with Trypan Blue (Vetec Ltda., Rio de Janeiro, Brazil) (0.05% in lactoglycerol) [43], and the gridline intersection method was used to estimate the percentage of colonization [44] (adapted). For the easily extractable fraction of GRSPs, the extraction method described by Wright and Upadhyaya [45] was used, and proteins were quantified using the Bradford [34] method.

The data were analyzed using the *t*-test ($p \leq 0.05$) in Assistat software (7.7). A Spearman correlation matrix, a decision tree, and clustering algorithm analyses (K-means) were computed for the evaluated parameters using Google Collaborative Platform.

3. Results

The season of sampling influenced the TPtC, TPC, TTC, TFC, and TPcC in *A. colubrina* leaves (Figure 3), showing a high correlation ($r \geq 0.6$; $p \leq 0.05$) with mycorrhizal colonization and GRSPs deposition, in dry and rainy seasons, respectively (Figure 4). Additionally, there was greater production of bioactive compounds in the leaves of *A. colubrina* individuals during the rainy season (Figure 3).

In this regard, the TPtC increased by 75% in individuals collected during the rainy season, compared to those in the dry season (Figure 4). The TPC, TTC, TSC, TFC, and TPcC in the leaves of individuals collected during the rainy season were, in general, about 100% higher when compared to the concentrations obtained from individuals sampled during the dry season (Figure 3). In this respect, it is worth noting that the TFC was a decisive parameter to differentiate the seasons (Figure 5), which is confirmed by the clustering algorithm analyses (Figure 6). TFC also showed increased biosynthesis during the rainy season, albeit to a lesser extent (approximately 25% higher than in the dry season) (Figure 3).

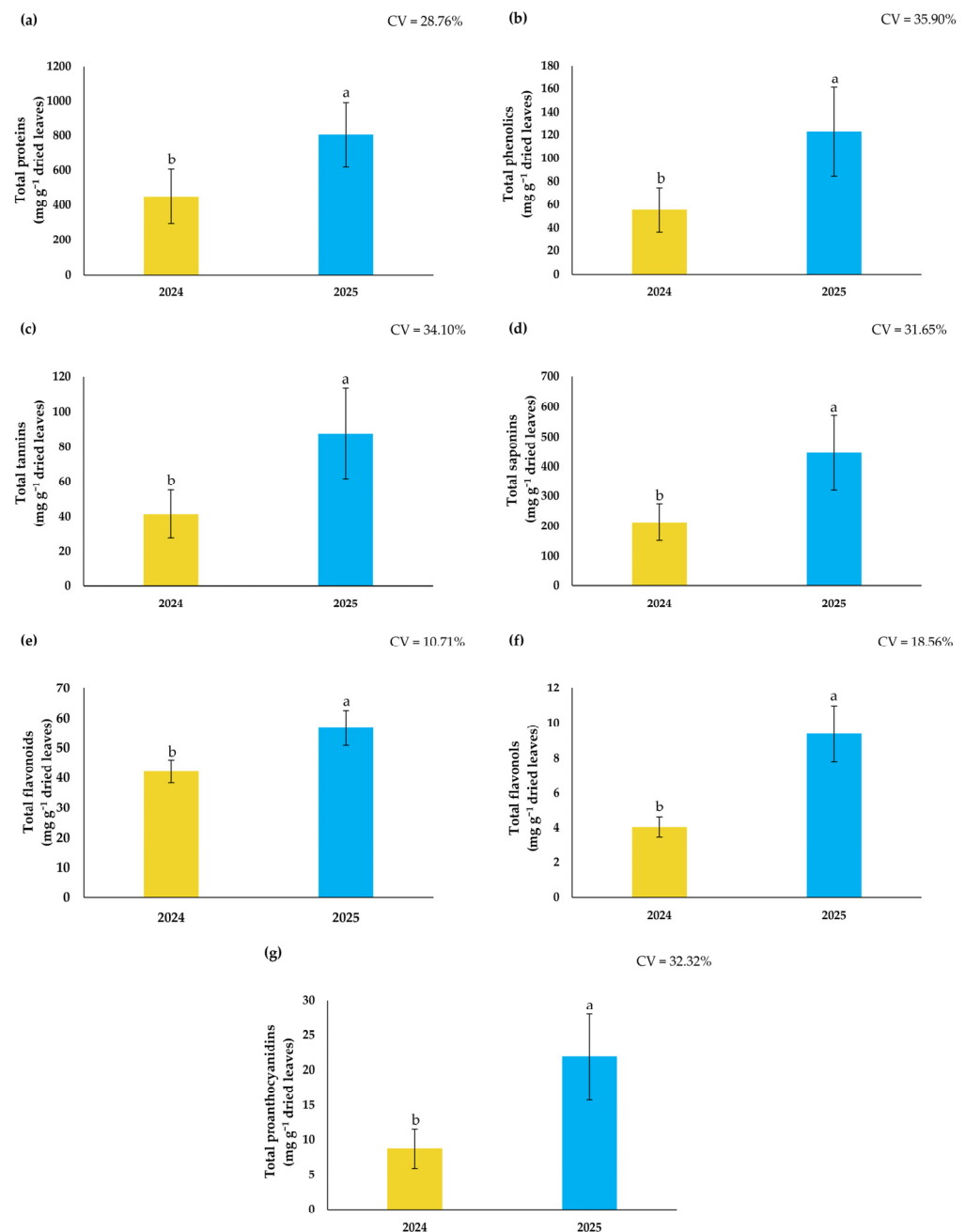


Figure 3. Concentration (mg g⁻¹ dried leaves) of total proteins (TPtC) (a), total phenols (TPC) (b), total tannins (TTC) (c), total saponins (TSC) (d), total flavonoids (TFC) (e), total flavonols (TFoC) (f), and total proanthocyanidins (TPcC) (g) in leaves of *Anadenanthera colubrina* (Vell.) Brenan, from a natural environment, during the dry season (collected in 2024, 9.2 mm of rainfall) and rainy season (collected in 2025, 178.5 mm of rainfall) ($n = 10$, for each season). CV (%): coefficient of variation. Means followed by the same letters do not differ significantly according to the t -test ($p \leq 0.05$). Error bars represent the standard deviation of means.

The mycorrhizal colonization and soil pH did not vary according to the season (Figure 7). Although these parameters did not vary significantly between sampling in the dry and rainy seasons, they were positively correlated with the TPtC, TPC, TTC, and TFC during the dry season (Figure 4a). Soil pH during the dry season was also correlated with mycorrhizal colonization (Figure 4a). Contrary to expectations, the concentration of GRSPs in *A. colubrina* rhizosphere was higher in the dry season, with approximately 30% reduction during the rainy season (Figure 7a). Despite this increase, the GRSPs deposition

was correlated with the TPcC and TPC of *A. colubrina* leaves harvested during the rainy season (Figure 4b).

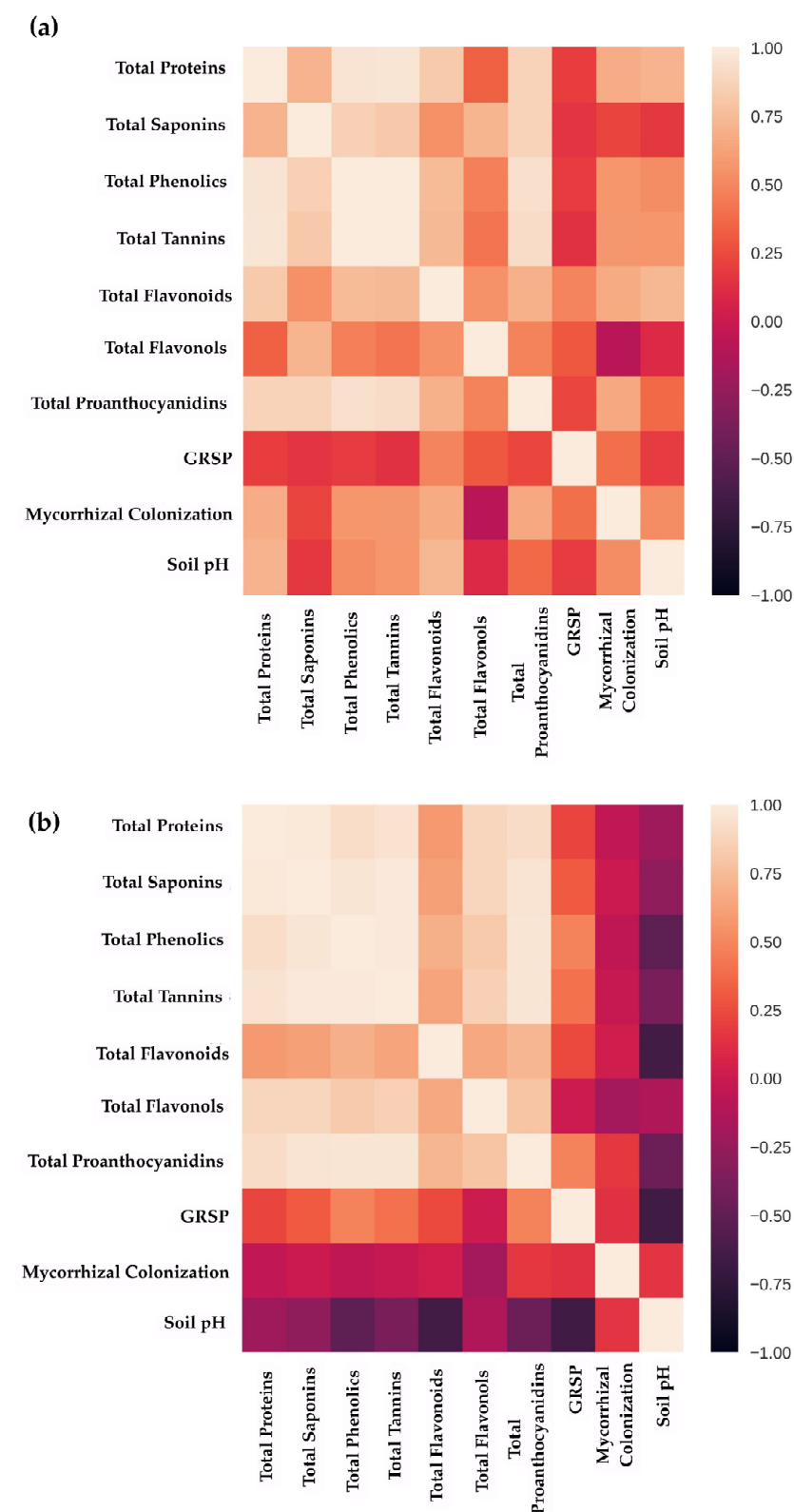


Figure 4. Spearman correlation matrix ($p \leq 0.05$) between the variables of total proteins (TPtC), total saponins (TSC), total phenolic compounds (TPC), total tannins (TTC), total flavonoids (TFC), total flavonols (TFoC), total proanthocyanidins (TPcC), glomalin-related soil proteins (GRSP), mycorrhizal colonization, and soil pH of *Anadenanthera colubrina* (Vell.) Brenan, from a natural population, during the dry season (a) and rainy season (b) ($n = 10$, for each season).

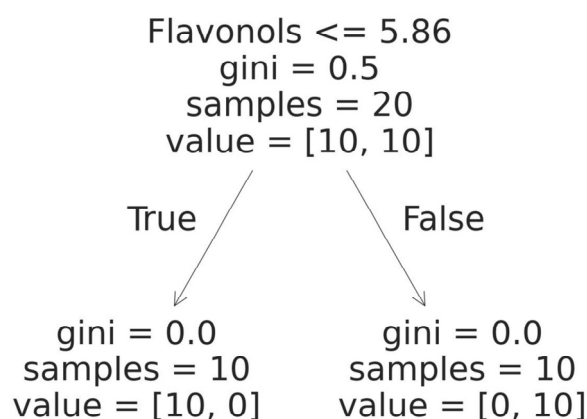


Figure 5. Decision tree considering the concentration of total flavonols (TFoC) in *Anadenanthera colubrina* (Vell.) Brenan, from a natural environment, during the dry season (collected in 2024, 9.2 mm of rainfall) and rainy season (collected in 2025, 178.5 mm of rainfall) ($n = 10$, for each season). True entropy values close to zero indicate maximum probability of the decision tree.

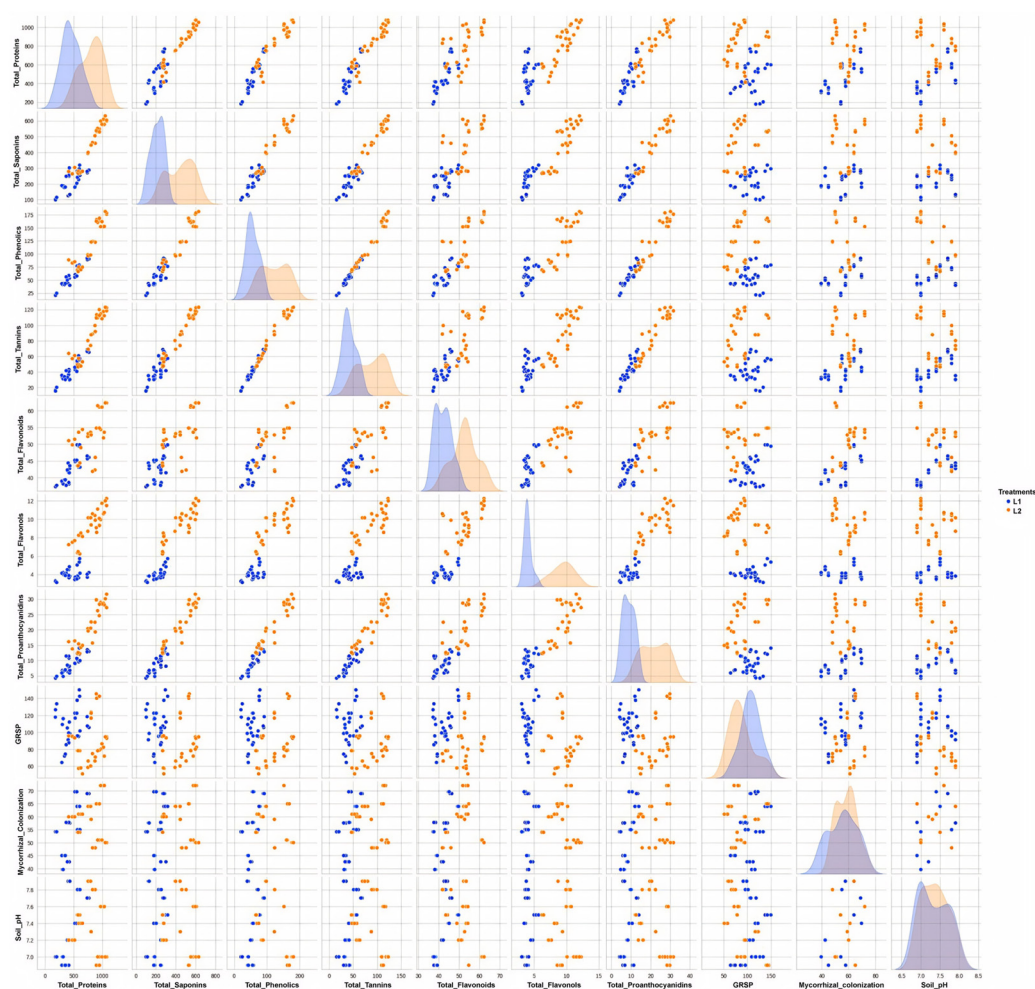


Figure 6. Data distribution by cluster considering the reduction in the total proteins (TPtC), total saponins (TSC), total phenols (TPC), total tannins (TTC), total flavonoids (TFC), total flavonols (TFoC), total proanthocyanidins (TPcC), glomalin-related soil proteins (GRSP), mycorrhizal colonization, and soil pH of *Anadenanthera colubrina* (Vell.) Brenan, from a natural environment, during the dry season (L1—collected in 2024, 9.2 mm of rainfall) and rainy season (L2—collected in 2025, 178.5 mm of rainfall) ($n = 10$, for each season), (K-means = 2).

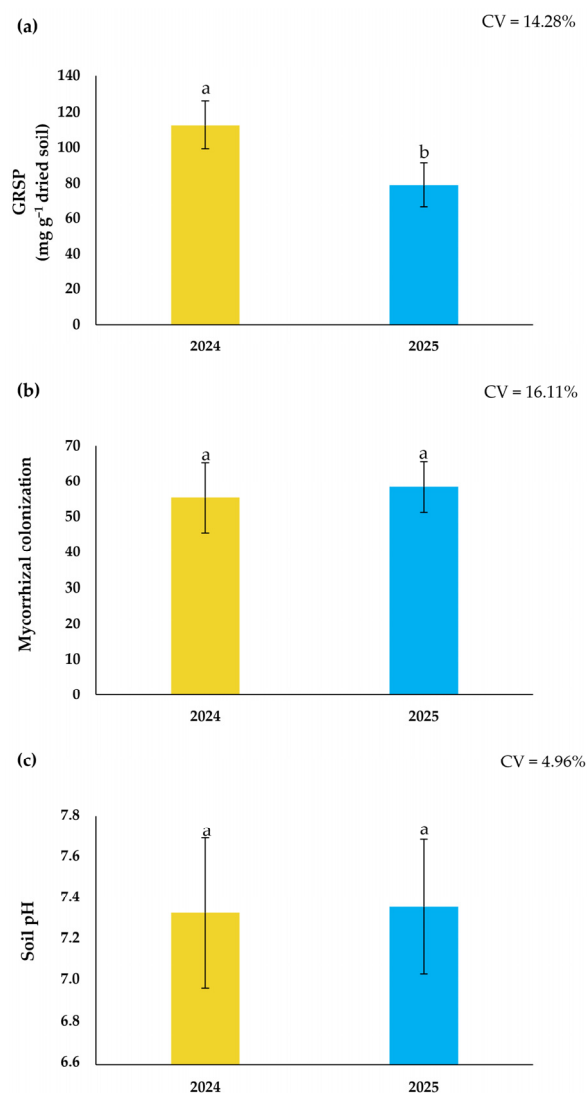


Figure 7. Concentration (mg g⁻¹ of dried soil) of glomalin-related soil proteins (GRSP) (a), mycorrhizal colonization (%) (b), and soil pH (c) of *Anadenanthera colubrina* (Vell.) Brenan rhizosphere, from a natural environment, during the dry season (collected in 2024, 9.2 mm of rainfall) and rainy season (collected in 2025, 178.5 mm of rainfall) ($n = 10$, for each season). CV (%): coefficient of variation. Means followed by the same letters do not differ significantly according to the t -test ($p \leq 0.05$). Bars represent the standard deviation of means.

4. Discussion

The protein values obtained in this study (451–805 mg g⁻¹ leaves) were higher than those reported by Pedone-Bonfim et al. [18] (200–310 mg g⁻¹ plant) for foliar proteins in mycorrhizal *A. colubrina* seedlings grown in a greenhouse. Similarly, the production of secondary metabolites obtained in the present study was also higher than that reported for seedling leaves of the same species grown under greenhouse conditions [17–21]. In addition, the values of total phenolics (55–122 mg g⁻¹ leaves) and total tannins (41–87 mg g⁻¹ leaves) were higher than those found in the bark (168 µg mL⁻¹ for phenols and 56 mg g⁻¹ for tannins) of individuals from other natural populations [25,46]. This pattern may be related to leaf renewal during the rainy season, since fluctuations in metabolite concentration in bark may have minor variations throughout the year [24–27]. The increased biosynthesis of specialized metabolites during the rainy season contrasts with that observed by Araújo et al. [24], who reported the greatest diversity and concentration of bioactive compounds in *A. colubrina* leaves during the dry season in a dry forest area in Brazil, the *Caatinga*. This

discrepancy may be attributed to site-specific differences, as metabolite production varies across phytogeographic domains [47].

The correlation found between the soil pH and mycorrhizal colonization during the dry season is consistent with the known ability of AMF to modulate soil parameters [20,48]. In this context, it is interesting to evaluate other parameters that may also be influencing the rhizosphere [49], such as the total GRSPs fraction and glomerospore production. It is also important to investigate the effects of deciduousness on mycorrhizal activity, a characteristic present in *A. colubrina* [27]. It is worth noting that *A. colubrina* has a high demand for nutrients [50], which is favored by mycorrhizal colonization [51]. This may explain the correlations of this parameter with the concentration of most bioactive compounds evaluated in leaves collected during the dry season (Figure 4a). In contrast, in the rainy season, this pattern was not observed (Figure 4b); the reduction in the AMF efficiency in environments with greater water availability [52] may explain the absence of correlation (Figure 4b). Moreover, the proportion of mycorrhizal structures in rainy seasons is something that needs to be elucidated in future research, considering that there may be greater stimulation for the formation of storage structures (vesicles) compared to those used for nutrient exchange (arbuscules) [53].

The reduction in the GRSPs concentration in *A. colubrina* rhizosphere is likely linked to higher accumulated precipitation (Table 1), considering that water accumulation in the soil can leach aggregates formed by GRSPs deposition [54]. On the other hand, the GRSPs correlation ($r \geq 0.6$; $p \leq 0.05$) with the production of some metabolites during the rainy season may be linked to their effect on soil structure [55,56]. In this case, we observed, for the first time, that rhizosphere GRSPs deposition is positively related to some products of tree foliar metabolism in natural conditions; this is relevant because it confirms the positive role of *A. colubrina*–AMF symbiosis, widely reported under controlled conditions [17–21].

From a practical standpoint, these results obtained in our study can be used by industries that include *A. colubrina* phytomass in the production of phytopreparations. In this matter, they can opt to use the leaves of the species, replacing the stem, since leaf tissues have a high concentration of bioactive compounds, such as phenolics and total tannins, being higher than those found in the bark [25,46]. This practice is more sustainable, given that leaves are a renewable resource, abundant throughout most of the year [27], and their use can reduce the excessive bark extraction, a practice that causes damage to plant populations [57,58]. However, it is recommended to collect leaves during the rainy season to obtain raw material with a higher accumulation of compounds that are important to the pharmaceutical industry [59,60].

5. Conclusions

Therefore, the study hypothesis was confirmed, since mycorrhizal colonization and GRSPs deposition were correlated with the production of foliar bioactive molecules in *A. colubrina* in a natural environment, highlighting the AMF role in foliar metabolite accumulation under natural conditions.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AMF	Arbuscular mycorrhizal fungi
APAC	Agência Pernambucana de Águas e Clima
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
FACEPE	Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco
HUFP	Geraldo Mariz Herbarium
LabFlora	Laboratório de Florística e Sistemática Vegetal
LAPEM	Laboratory for Analysis, Research, and Studies in Mycorrhizae
TFC	Total flavonoids
TFoC	Total flavonols
TPC	Total phenols
TPtC	Total proteins
TPcC	Total proanthocyanidins
TTC	Total tannins
CEC	Cation Exchange Capacity
CV	Coefficient of variation
GRSPs	Glomalin-related soil proteins
SisGen	Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado
UFPE	Federal University of Pernambuco
UPE	University of Pernambuco

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