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Quantification of tannins in *Anadenanthera colubrina* (Vell.) Brenan seedlings and its relationship with environmental and agronomic variables

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TECHNOLOGY OF FOREST PRODUCTS

ABSTRACT

Background: *Anadenanthera colubrina* (Vell.) Brenan, commonly known as angico, is a species of high socioeconomic and environmental value, distinguished by its high concentration of tannins in various plant parts. This study aimed to co-validate the quantification method of tannins and associate their quantification with data on the development of young specimens of *Anadenanthera colubrina* (Vell.) Brenan from different environments. Seeds were collected in two areas in the Western Cariri of Paraíba. Plants were evaluated regarding their height, diameter, fresh and dry mass, and tannin quantification 90 days after emergence. Partial validation was performed in accordance with current Brazilian legislation.

Results: Regarding the development data, only height was statistically different between the study areas, and the specimens from Sumé were the highest (73.6 cm) ones. The tannin quantification method proved to be selective, linear, accurate, and robust, with analytical reliability in the quality control of the plant drug the of part aerea. Principal component analysis justified 79.3% of the data, evidencing the efficiency of the adopted model. Two main groups were formed, in which biomass and diameter data were associated with specimens of matrices from Serra Branca, whereas specimens from Sumé were more related to tannin production and plant height.

Conclusion: This set of data contributes to the sustainable use of the species and is promising in the innovation and technological development of sustainable products based on raw materials rich in tannins from *A. colubrina*.

Keywords: Analytical Technologies; Caatinga; young specimens; UV-VIS spectrophotometry.

HIGHLIGHTS

Plants from municipalities in the same Microregion of Paraíba present different tannin levels
The origin of the seeds influenced the growth and variation of the studied seedlings.
Innovative model replaces the extractive use of *Anadenanthera colubrina* with cultivated seedlings.
Advances Sustainable Management of *Anadenanthera colubrina*.

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INTRODUCTION

Anadenanthera colubrina (Vell.) Brenan, commonly known as "angico," is a species belonging to the Fabaceae family, native to Brazil. It is found from the Northeast to the South of the country and is distributed across the Caatinga, Cerrado, and Atlantic Forest biomes (Bispo et al., 2017). This species holds significant socio-economic and environmental value within the Caatinga biome (Pessoa et al., 2012) and stands oufor its high tannin in various plant parts, which plays, playing a critical role in plant defense and having numerous applications in the pharmaceutical, food, and tanning industries (Melo et al., 2010).

To ensure the quality of phytotherapeutic products, it is essential to standardize production processes and characterize both plant inputs and final products through quantitative analyses of chemical markers in the plant material (Donno et al., 2016), as well as for any phytoproduct. Studies have shown that environmental and agronomic variables during the production and development cycles of the plants also influence secondary metabolite production, necessitating the characterization and standardization of these species (Simões et al., 2010; Gomes et al., 2021a). Analytical method validation is essential to ensure the reliability of the information about analyzed samples, meeting the requirements for analytical applications (Brazil, 2017; Silva et al., 2020).

In addition to the unsustainable removal of bark for medicinal purposes, in just one tannery in Cabaceiras, in the Cariri region of Paraíba, the consumption of bark from this species is approximately 200 tons per year (Matos Júnior et al., 2017). Once it is proven that it is economically viable to replace the bark of adult individuals with young individuals for tannin production through validated methods, there will be a great contribution to the conservation of the species and the sustainable economic development of the region.

This study aimed to co-validate the quantification method of tannins and associate their quantification with data on the development of young specimens of *Anadenanthera colubrina* (Vell.) Brenan from different environments.

MATERIAL AND METHODS

Seed collection and plant production

Seeds of *A. colubrina* were collected from adult matrices located in two municipalities in the Western Cariri region of Paraíba, in 2019. The collections were carried out specifically in the rural area of Serra Branca (Paraíba, Brazil) [07º 30' 51.1" latitude (S) and 36º 41' 91.5" longitude (W), average altitude of 493 meters] and Sumé (Paraíba, Brazil) [07º 40' 18" latitude (S) and 36º 52' 48" longitude (W), average altitude of 533 m] (Brazilian Institute of Geography and Statistics [IBGE], 2019).

The collected seeds were taken to the Laboratory of Ecology and Botany of the Center for Sustainable Development of the Semiarid, Federal University of Campina Grande (LAEB/CDSA/UFCG), in the municipality of Sumé, where they were processed manually. The production and monitoring of plant development were performed in the seedling nursery of the LAEB/CDSA/UFCG, in October 2019. One hundred seeds from each municipality were sown in seedling bags measuring 23 cm in height by 11 cm in width. The substrate used was composed of sand, manure, and subsoil in a ratio of 1.5:1.0:0.5. Each seedling bag was irrigated daily with 150 ml of water.

The development of the specimens was evaluated 90 days after emergence by determining the height of the aerial part, measuring the distance between the collar and the apex, using a centimeter-graduated ruler, and the stem diameter at ground level using a digital caliper (0.01 mm). Fresh and dry masses were also evaluated. A precision scale (0.0001 g) and a forced air circulation oven, at 42 °C until constant mass, were used. The results were expressed in g/seedling.

Collection and processing of the plant drug the of aerial part

90 days after emergence, the aerial parts of 15 specimens from each municipality were collected, representative of the total sample, with a height of 60 – 86 cm, for tannin quantification. The collected samples were dried in an air circulation oven at 42 °C until a constant mass was achieved, ground in an analytical mill Q298A (Quimis, Brazil), homogenized according to the area, and subjected to a sieve system in an industrial sieve, retaining particles with granulometric <50 mesh.

Extraction, preparation, and reading of solutions

Extraction was performed using an IKA RW 20 propeller mechanical stirrer at 720 Hz/min for 10 minutes. A 50% hydroalcoholic extractive solution was used. Five concentrations of the plant drug the of part aerea (0.750, 1.125, 1.500, 1.875, and 2.250 g) were evaluated, which were diluted in 50 ml of the extractive solution. The samples were filtered through filter paper, using a vacuum pump, and completed in a volumetric flask (50 ml). Three replicates were performed for each sample.

Tannin content was determined by the vanillin reaction method: HCL, in which 200 µl of the extract was pipetted from the average concentration (1.5 g of plant drug the of part aerea for 50 ml of 50% hydroalcoholic solution) + 1.5 ml of a 4% vanillin solution, initially diluted in methanol and then diluted again in PA HCl (37%). The reaction occurred in test tubes for 20 minutes; afterward, 1.3 ml of distilled water was added to complete the final volume of 3 ml. The readings were made in an ultraviolet-visible spectrophotometer at 500 nm against a blank.

Partial validation

The following parameters were evaluated in the partial validation: selectivity, linearity, limits of detection and quantification, accuracy, and robustness (Brazil, 2017). These parameters were evaluated in plant extracts from Sumé.

Selectivity – The selectivity of the analytical method was demonstrated by its ability to unequivocally identify or quantify the analyte of interest in the presence of components that may be present in the sample, such as impurities, diluents, and matrix components, obtaining a positive result from the sample containing the analyte and a negative result for other substances in the sample (Brazil, 2017). The selectivity of the method was evaluated through the application of the Scan method, with scanning from 400 to 800 nm in an ultraviolet-visible spectrophotometer to identify the tannin peak in the catechin pattern and the aerial part extract. The working solution for the plant drug the of part aerea and catechin were 30 mg/ml and 20 µg/ml, respectively.

Linearity – The quantification of tannins was performed based on catechin curves developed by (Gomes et al., 2021b), for young specimens of *Sideroxylon obtusifolium* (Roem. & Schult.) T.D. Penn. The following solution concentrations were obtained for tannins: 26.5, 39.8, 53.1, 66.4, and 79.6 $\mu g.ml^{-1}$. The curves were prepared on three different days. To verify the linearity (the method's ability to generate results directly proportional to the concentration of the analyte), the data were subjected to linear least squares regression, obtaining the straight-line equation and the minimum acceptable correlation coefficient $R^2 = 0.991$ (Brazil, 2017).

Limits of Detection and Quantification – To calculate the limits of detection and quantification, the values were estimated in µg/ml considering the relationship between the standard deviation and the angular coefficient (slope of the straight line) obtained by linearity. Equations 1 and 2 were used to determine the limits of detection and quantification, respectively (Brazil, 2017).

$$LD = 3.3.\ s / IC \quad (1)$$

$$LQ = 10.\ s / IC \quad (2)$$

Where LD is the limit of detection, LQ is the limit of quantification, IC is the slope of the calibration curve, and s is the standard deviation.

Accuracy – Intermediate accuracy was evaluated, aiming to assess the proximity between the results obtained from the analysis of the same sample, in the same laboratory, on two different days, contemplating the same concentrations. Six individually prepared replicates at 100% test concentration were used. Accuracy was demonstrated by the dispersion of results, calculating the relative standard deviation (RSD) of the series of measurements and Tukey's test at 5% significance (Brazil, 2017).

Robustness – The test to determine robustness was performed by preparing the samples considering the stability of the extractive solution at time 0 (immediate reading) after 48 and 72 hours. The fluid extract samples were stored under refrigeration.

Quantification of tannins in plant drugs the of part aerea from Sumé and Serra Branca

After partial validation, plant drugs the of part aerea from Sumé and Serra Branca were quantified according to the method already described, with accuracy data expressed in mg/g for comparison between the two municipalities. Multivariate analysis was applied through principal component analysis via a correlation matrix to understand the clustering between the different parameters analyzed. The values of each parameter were previously normalized through autoscaling so that the variables obtained the same importance (weight).

Statistical analysis

The statistical design of plant production was completely randomized and the data were subjected to analysis of variance (ANOVA), with four replications. Means were compared by Tukey's test at 5% probability. In the quantification of tannins, the arithmetic means, standard deviation, and coefficient of variation (CV%) were calculated. The results were obtained using the Excel Action Stat Pro, version 3.6, and the graphs were generated in the Origin Pro 8 software.

RESULTS

The data referring to the mean height, diameter, fresh mass, and dry mass of *A. colubrina*, 90 days after emergence, are shown in Table 1. Only the height differed statistically between the two study areas.

The selectivity of the analytical method was demonstrated through its ability to unequivocally quantify the tannin content, in the presence of components in the samples, ensuring a peak response of the catechin and the *A. colubrina* extract at a wavelength of 500 nm (Figure 1).

The calibration curves used for linearity were developed by Gomes et al. (2021b). In the method developed by these authors, catechin had a linearity range from 10 to 80 $\mu g.ml^{-1}$, which generated a linear regression equation for the mean curve of $y = 0.0142x + 0.0178$ ($R^2 = 0.999$).

Table 1: Development of *Anadenanthera colubrina* specimens of matrices from different environments, 90 days after emergence.

Environment	Height (cm)	Diameter (mm)	Fresh mass (g)	Dry mass (g)
Sumé	73.6 a ± 7,0	3.78 a ± 0,7	15.2500 a ± 3,6	6.7338 a ± 1,75
Serra Branca	65.96 b ± 7,0	3.76 a ± 0,4	14.7512 a ± 3,9	6.4559 a ± 1,79

Means followed by the same letter in the column do not differ statistically by Tukey test at 5% probability.

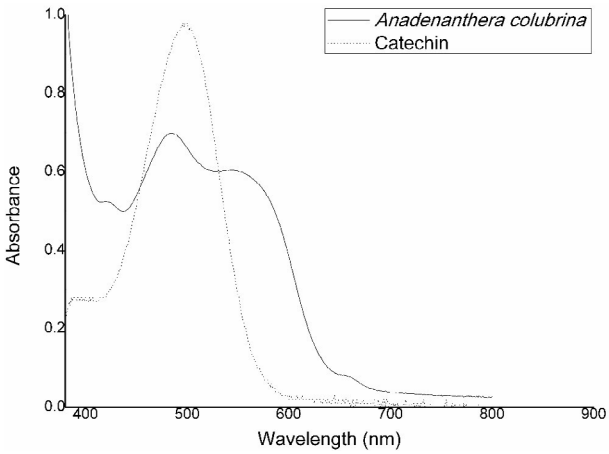

Figure 1: UV-Vis spectra of the standard solution of condensed tannins (Catechin) and the *Anadenanthera colubrina* extracts.

The linearity of the plant drug the of part aerea was verified by the analytical curve of fluid extracts of tannin concentration from *A. colubrina*, in their concentration ranges. The mean linear regression equation obtained from the calibration curves was y = 0.0115x + 0.1875, where y is the absorbance and x is the concentration (µg/ml) equivalent to the fluid extracts of tannin concentration. The coefficient of determination was R^2 = 0.9986, for fluid extracts of tannin concentration (Figure 2).

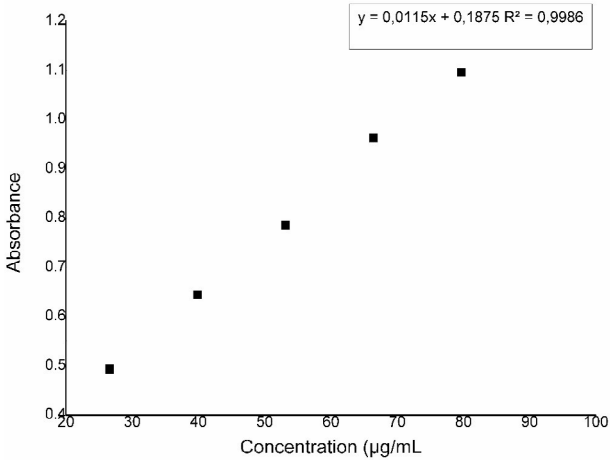

Figure 2: Linearity of fluid extracts of tannin concentrations in *Anadenanthera colubrina*.

The limit of detection (LD) and limit of quantification (LQ) for tannins in *A. colubrina* were 9.067 µg/ml and 27.476 µg/ml, respectively (Table 2).

The intermediate accuracy in fluid extracts samples demonstrated that, regarding repeatability, the method corroborates the parameters required by current legislation, showing a relative standard deviation (RSD) of less than 5% and no statistical difference by the Tukey's test at 5% probability, between the analyzed days (Table 3).

The robustness evaluation of the fluid extract showed that there was no change in the stability of the samples in the evaluated periods, when stored under refrigeration, given that the concentration values of *A. colubrina* had no significant variation ($P<0.05$) during the period of sample storage, showing that the method used was robust (Table 4).

Regarding the tannin quantification in young specimens of *A. colubrina* matrices from the municipalities of Sumé and Serra Branca, tannin concentrations were 28.05 ± 3.5 (mg/g) and 26.55 ± 1.7 (mg/g), respectively, statistically differing between the study areas (Table 5).

The first principal component explained 50.4% of the data total variation, in which the development variables (height, diameter, and dry mass) were mainly characterized by the scores of the specimens from Sumé. The second principal component justified 28.9% of the data, which was also better characterized by the specimens from Sumé, especially regarding the quantification of tannins. The specimens from Sumé had a positive association between tannin production and plant height, whereas the specimens from Serra Branca had a greater correlation with the diameter and dry mass, thus obtaining a negative correlation with tannin production (Figure 3).

DISCUSSION

The data relating to the means of development of *A. colubrina* specimens of matrices from Sumé and Serra Branca showed little difference between the areas of seed collection, differing only in the height parameter. This may be related to environmental factors, as both municipalities are located in the Western Cariri region of Paraíba. However, the substrate and 50% shading showed efficiency regarding the height, diameter, and aerial biomass development, since the plant production parameters had a good performance during the 90 days of evaluation.

Regarding plant height, 90 days after emergence, our research obtained significant values both for specimens from Sumé (73.6 cm) and Serra Branca (65.96 cm) when compared to values found in the literature. Brondani et al., (2008), evaluating *A. colubrina,* 95 days after germination, obtained

Table 2: Limits of detection and quantification (µg/ml) for the concentration of tannins in *Anadenanthera colubrina*.

Solution	Standard Deviation of Residues	Angular coefficient	Detection limit µg/mL	Quantification limit µg/mL
A. colubrina	0.0314	0.0115	9.067	27.476

a maximum height of 17.2 cm with a maximum technical efficiency dose (MTED) estimated at 2743 mg dm^{-3} of fertilizer.

Table 3: Intermediate accuracy of tannin concentration in *Anadenanthera colubrina*.

Factor	Averages concentration (µg/mL)	Standard deviation (%)
Day 1	52.66 a	2.04
Day 2	53.58 a	0.76

Means followed by the same letter in the column do not differ statistically by Tukey test at 5% probability.

Table 4: Tannin robustness tests in fluid solutions of *Anadenanthera colubrina*.

Stability (hours)		
Time	Average concentration (µg/mL)	Standard deviation(%)
0	52.66 a	2.04
48	53.58 a	0.76
72	52.77 a	1.83

Means followed by the same letter in the column do not differ statistically by Tukey test at 5% probability.

The diametric development of specimens from the two study areas ranged from 3.76 to 3.78 mm. These values are higher than those reported by Brondani et al. (2008), who found a value of 2.12 mm, with an estimated dose of 1513 mg dm-3 of fertilizer approximately 92 days after germination. In addition, these authors observed a decrease in this variable at 95 days, with 2 mm at a dose of 5000 mg dm^{-3} of fertilizer. According to Pereira et al. (2017), the height of the aerial part combined with the collar diameter, as well as the dry biomass, are good quality indicators of seedlings of forest species.

Table 5: Concentration of tannins (mg/g) in young specimens of *Anadenanthera colubrina* from different municipalities in the Cariri region of Paraíba.

Environment	Concentration of tannins (mg/g)
Sumé	28.05 ± 3.5a
Serra Branca	26.55 ± 1.7b

Means followed by the same letter in the column do not differ statistically by Tukey test at 5% probability.

According to Rossa et al. (2015), analyzing the fresh and dry mass of *Anadenanthera peregrina* (L.) Speg, found maximum values of 6.56 g and 3.58 g, respectively, 189 days after sowing, in an experiment conducted in a nursery, using a mixture of organic compost, vermiculite, and Plantmax® as substrate. Both masses had basically half of the values recorded for *A. colubrina* in our research. In addition to being different species, biomass production is closely associated with the physiological quality of seeds, maturation, and environmental variables.

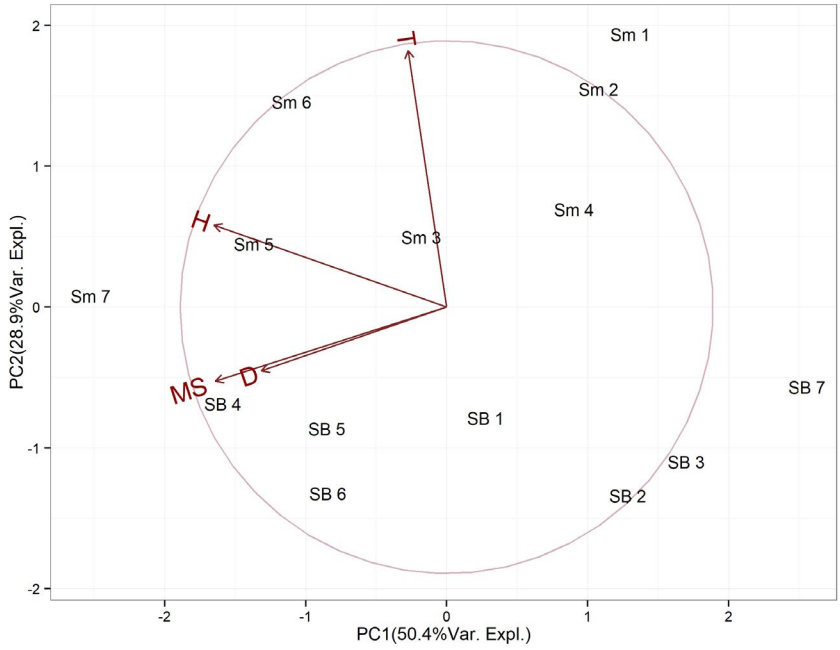

Figure 3: Principal component analysis of tannin production and development of young specimens of *Anadenanthera colubrina* from matrices in different environments.

Total or partial analytical validation is extremely important in quality control, being a determining analytical method for the results. Selectivity is the first step in the development and validation of analytical separation methods. According to Cardoso et al. (2010), the selectivity of an analytical method evaluates not only the presence of the active compound under study but all related compounds that may be normally present and that may interfere with its determination in a complex sample. The selectivity results for the extracts of *A. colubrina* ensure a peak response exclusively from the active principle, i.e., from the compound of interest, with no interferences in the sample. The peak was recorded at a wavelength of 500 nm, within the wavelength of the standard catechin solution, thus ensuring the selectivity of the method.

To ensure the quality control of several pharmaceutical products, including phytotherapics, the ultraviolet spectrophotometry technique is widely used due to its speed, low operational cost, and high reliability of results (Borba et al., 2013). According to Iammarino et al. (2017), selectivity is the parameter responsible for the certainty of identification by the method, for the analyzed response variable, differing the analyte in the presence of other components, thus indicating the impurities and interferents that may lead to false identifications in the studied method. Thus, this parameter should be continuously checked during the tests for the other parameters (Goes Junior et al., 2019).

The linearity of the method proved that more than 99% of the experimental variability can be explained by linear models and that the method is adequate regarding the relationship between analyte concentrations and spectrophotometric responses. According to ANVISA Resolution 166 (Brazil, 2017), the coefficient of determination (R^2) must be above 0.991, meaning that there is little dispersion between the obtained values and less uncertainty. According to Brito et al. (2003), linearity refers to the ability of the method to generate results linearly proportional to the concentration of the analyte, in the specified analytical range, in which this parameter can be demonstrated by the correlation coefficient of the analytical curve.

The detection limit indicates the smallest value of a variable (concentration) that can be detected but not necessarily quantified for the accuracy and precision limits required by the method, and the quantification limit indicates the smallest possible quantification for a variable in a given evaluated method (Cassini et al., 2013). All partial validation and quantification of tannins were within the limit allowed both for detection and quantification. Therefore, the methodological procedure provided spectrophotometric responses with sensitivity for detecting and quantifying tannins in *A. colubrina* extracts with the desired reliability, thus showing that the analytical method has high sensitivity.

The intermediate accuracy results, in addition to presenting a relative standard deviation of less than 5%, showed no statistically significant difference between samples analyzed on different days, indicating that the method can be reproduced. The accuracy of the analytical method determines the degree of agreement between the results of independent measurements around a central value, being performed several times on a homogeneous sample. Accuracy is considered one of the most representative analyses because it reveals the effect of variations related to the days analyzed, implying the guarantee of the method's reproducibility (Rubim et al., 2012).

Robustness is assessed through a small variation of known parameters, verifying its influence on the response, and corresponds to the ability of a method to resist small changes in the analytical parameters, indicating its reliability during its normal use (Brazil, 2017). According to Borba et al. (2013), good robustness indicates that the method remains reliable and accurate in the face of oscillations. The evaluated method proved to be robust because the extracts remained stable when stored under refrigeration for 72 hours.

A. colubrina specimens of matrices from Sumé stood out with a higher concentration of tannins in comparison with those from Serra Branca. There is no record in the literature on the quantification of tannins in young individuals of this species; the studies are focused only on adult individuals. This may be due to the use of this species only in the adult stage based on extractivism.

According to Gomes et al. (2021b), evaluating specimens of *Sideroxylon obtusifolium* (Roem. & Schult.) in the municipalities of Sumé and São João do Cariri, both in the Cariri region of Paraíba, observed that the aerial part of specimens from Sumé had 70.43 mg/g of tannins. According to these authors, the aerial part contains up to 61% more tannins than the stem periderm, making it possible to replace the traditional use of the stem periderm with the use of the aerial part. These authors also highlighted the influence of phenology and different particle sizes on the quantification of tannins, since specimens with low phenological reproductive intensity throughout the year had a higher concentration of tannins in the smallest particles of the aerial part.

For Gobbo-Neto and Lopes (2007), the age and development of the plant, as well as the different plant organs, are also of relevant importance and can influence the total amount of secondary metabolites. The concentration of condensed tannins is controlled firstly by genetic factors and then by environmental variables and is associated with the tissue lignin concentration, which tends to increase with plant maturity (Acuña et al., 2008). In this sense, Gebrehiwot et al. (2002) explain that the concentration of condensed tannins changes considerably depending on the species, stage of development, and soil fertility.

According to Gomes et al. (2021b), there is a significant influence of different specimens in the quantification of tannins, which are directly related to the environments the specimens are found. In addition to environmental characteristics, the development cycle and plant management also influence the production of tannins (Gomes, 2021). Corroborating this information, the principal component analysis of *A. colubrina* showed the formation of two main groups, in which the plant development data (biomass and diameter) are associated with specimens from Serra Branca, whereas specimens from Sumé have a greater relationship with tannin production and height development. In general, the data clustering by the similarity of 79.3% was justified, evidencing the efficiency of the adopted model.

In his research, Cruz (2019) observed that the quality of *A. colubrina* seeds from *A. colubrina* influenced the initial performance of the produced seedlings and that this influence was directly related to the harvest area, showing dissimilarity between the quality of seedlings originating from seeds collected in different places, where for the seedling quality parameters, 63.82% of the variability was explained by the principal component 1 and 36.18% by the principal component 2. According to this author, the evaluated parameters were sensitive for the discrimination of differences between the groups, since attributes that indicate the quality of seedlings, such as plant height, collar diameter, and dry matter of the aerial part, were attributed to the origin from two environments.

CONCLUSION

The place of origin of *A. colubrina* seeds influenced the physiological potential, as well as the development of seedlings, indicating that the variations are responses of the species' adaptations to a certain environment, due to the interaction between genotype and environment; even though they are from the same species, individuals have different performances.

The quantification of tannins in young specimens of *A. colubrina*, considering the origin from different environments associated with partial validation of the method and the association with development data, brings an innovative model regarding the possibility of replacing the extractive use with the use of cultivated individuals. Therefore, the set of data contributes to the sustainable use of the species, being promising in the technological development of new sustainable products based on raw materials rich in tannins from *A. colubrina*.

AUTHORSHIP CONTRIBUTION

Project Idea: AVL; ROM; ACG; LBM

Funding: AVL; ROM

Database: LBM; ACG; AVL; ROM

Processing: ACG; LBM

Analysis: LBM; ACG; AVL

Writing: LBM; ACG

Review: AVL; ROM

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