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High-throughput phenotyping by applying digital morphometrics and fluorescence induction curves in seeds to identifying variations: A case study of *Annona* (Annonaceae) species

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ABSTRACT

Differences in physical and structural characteristics of seeds may indicate variability within and between plant populations. In the present study, we performed a close characterization of dimension, shape, and tegument delayed chlorophyll fluorescence in seeds obtained from three species of the genus *Annona* (Annonaceae), i.e., *Annona coriacea*, *A. montana*, *A. squamosa*. Results showed that studied seeds may be sorted as scalene ellipsoids expressing low values for the seed sphericity. The morphological estimates suggested differences in seed shape for all species. A high correlation was observed between surface area and volume ($r^2 > 99\%$) for all the three species suggesting that in addition to structural shape. In addition, we also observed very high positive correlations (Rho = 1.000, $p < 0.001$) between surface area and arithmetic mean diameterof the seeds for all species. The first principal component (PCA1) of elliptical Fourier descriptors explained most of the variations in morphological structure of the seeds in the three species. Additionally, a less

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Abbreviations: EFD, elliptic Fourier descriptors; FT, Fourier transform spectra; K, kutorses; Ø, sphericity; Ra, aspect ratio; S, asymmetry; S_A, surface area; ST, short-time Fourier transform; V, seed volume; Λ, total amplitude; σ^2, variance; $\bar{x}$, mean

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intense tegument delayed chlorophyll fluorescence was observed for *A. montana* while the highest intensity was recorded for *A. squamosa*, revealing the potential use of fluorescence spectroscopy in discrimination at the species level by analyzing the frequency domain by means of Fourier Transform spectra as well as the relationship time-frequency of chlorophyll fluorescence.

1. Introduction

Quantification of biological parameters by mathematical models and statistical methods obtained from experimental data is a practice which is widely established in various branches of biology [1]. Morphometric discrimination of seeds presents an important tool for intra- and interspecific detection of differences in morphology with taxonomic, phylogenetic, and ecological purposes; for example, determination of differences among plant populations [2,3]. This is considered as a basic parameter which helps in understanding of dispersal syndrome and successional stages, providing additional data for studies on physiology of germination in natural regeneration programs or in restoration of the vegetation coverage in deteriorated areas [4,5]. In recent years, morphometric analysis (*i.e.*, length, width, and thickness) has been used to detect and interpret the differences in shape and size of seeds [6–8], allowing a better understanding of the relationship between individuals and groups [9].

The effect of variability on size, genetic, physical, and physiological quality of seeds, were the target of several studies with different approaches, considering different performance components in seeds and their resultant seedlings [10,12]. Morphological features of seeds are important to design and build pre-processing, processing, and classification machines in mechanized agricultural systems. In addition, the seed morphological features are essential for designing of storing bulk materials [13]. Linear dimensions of orthogonal axes have been widely used in the morphometric characterization of seeds [2]. However, there are few reported studies on measuring seed physical properties in phylogenetic studies [14–16], and such data can be organized in dimensional (shape, volume, surface area, sphericity, aspect ratio, density, moisture) or thermodynamic (energy dissipations) features.

Molecules exposed to an incident light beam (photons) may absorb and then dissipate the excess of absorbed energy by radiative processes (*i.e.*, fluorescence and/or phosphorescence) and/or non-radiative processes (i.e., vibrational and/or rotational decays) [17]. It is well established that fluorescence signal can be used to study several chemical, physical, and biological agents [18]. In fact, chlorophyll fluorescence (ChlF) has been routinely used for monitoring the physiological status of plants under stress [19–21]; for instance, ChlF was recently applied for the classification of seed vigor [22,23].

Despite being a widely used method in the quantification of leaf stress, the ChlF analysis has been limited to the time domain. However, there are two main ways to obtain the time-resolved fluorescence. One possible way is exciting the sample with a light pulse and collecting the fluorescence as a function of time, in the case of ChlF, commonly measured by prompt ChlF (OJIP-rise). In another way, the sample is excited with modulated light and then the fluorescence, as a function of time, is obtained from the frequency response of the ChlF emission, usually using a pulse-amplitude-modulated fluorimeter (PAM). In both cases, the goal is to get the parameters that describe the fluorescence as a function of time [21–24]. The time resolved ChlF can contain more information than the steady state ChlF in which it potential has not been fully explored yet, reinforcing the importance of developing other methods of analysis.

For most vegetable species, the fluorophores content in the seed coat decreases during maturation stages due to the degradation of the pigments such as chlorophylls, carotenoids, proplastids, and anthocyanins [7,25]. The relationship between amount of chlorophyll and seed maturity has been well studied in cabbage (*Brassica oleracea* L.) and wild turnip (*B. rapa* L.) seeds [22,25]. However, there are scarce information in the literature on ChlF in seeds, or it's potential in discriminating multiple species.

The Annonaceae family, with Pantropical distribution, has a wide ecological diversity among its species, with about 130 genera and 2.200 species [26,27] among a significant number of fruit species with high economic values [28]. In Brazil alone 29 genera and 392 species are cataloged [29]. The seeds of the Annonaceae family are considered one of the most uniform seeds in terms of morphological structure, and are albuminous ellipsoids in an obovoid format, featuring ruminations from the fibrous tegument inserted in the endosperm [27]. These ruminations are striking characteristics of this botanical family, associated with the diminute embryo; being critical features in terms of taxons at the family level. As reported by Setten and KoeK-Noorman [30], the seeds of Annonaceae have undeveloped embryos, are considered as immature, and they need time to complete their development and germination cycles.

Annona is the second largest genus of Annonaceae, and includes approximately 166 species of trees and shrubs [29]. In the Brazilian savannah, several *Annona* species are found, with ecological and economic importance due to their fruits [31].

Although it considered as a family with more uniform characteristics, both on anatomical and structural point of view and in terms of habit and habitat [30,32], the species of *Annona* are basically propagated through seed germination in a very slow and non-uniform process. In addition, there is

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scarce information about the dimensional profiles of the seeds, both at the genus and family levels. As a general rule, the identification of *Annona* species is carried out by planting the seed and waiting for the germination and development of plants, and it can be identified based on a morphological traits. However, time-consuming and cannot be easily employed for routine identifications for species with economic potential. An alternative can be the use of analytical methods based on non-destructive seed traits with appropriate modeling. Thus, in the present study, we propose a non-destructive and simple method to distinguish and characterize the morphological standards using the structural patterns of seeds, based on the linear dimensions of orthogonal axes as well as the ChlF emission of seeds from three species of the genus *Annona*.

2. Experimental setup and procedure

2.1. Plant materials

Material from three species of the *Annona* genera (Annonaceae) were analyzed in this study: *Annona coriacea* Mart., *A. montana* Macfad., and *A. squamosa* (L.) Bark (Fig. 1).

Infructescences of *A. coriacea* and *A. montana* were collected from different areas selected in Brazilian Savannah (22°05′48.2″S and 55°15′55.1″W) located in the State of Mato Grosso do Sul – Brazil. *A. squamosa* were collected from naturally ripened pods of intact plants growing in a pasture area at Zona da Mata of Minas Gerais, Southeast region of Brazil (21° 33′51.29″S and 42°26′58.44″W). The mature infructescences were put into plastic trays covered with filter paper for drying and subsequent manual processing of seeds. The seeds were separated, placed into labeled glass tubes and kept under refrigeration until the data measurement. The botanical identification was performed by Professor Dr. Etenaldo Felipe Santiago at the State University of Mato Grosso do Sul, and voucher specimens was deposited in the herbarium of the Universidade Federal da Grande Dourados (Table 1).

2.2. Structural and physical characteristics of seeds

The seed size was determined picking out randomly 100 seeds and measuring its three principal dimensions according to its morphology, namely, major, medium and minor axes using a digital caliper with an accuracy of 0.01 mm. The mean diameter of the seed, arithmetic mean (D_a), geometric mean (D_g) and specific mean (D_e) was calculated according to Sahay and Singh [33]:

$$D_g = (abc)^{1/3} \quad (1)$$

$$D_e = \left[a\frac{(b+c)}{4}\right]^{1/3} \quad (2)$$

$$D_a = \frac{(a+b+c)}{3} \quad (3)$$

The surface area of seeds was found by analogy with a sphere of same geometric mean diameter, using the expression cited by McCabe et al. [34]:

$$S_A = \pi D_g^2 \quad (4)$$

The aspect ratio (R_a) was obtained using the expression as recommended according Varnamkhasti et al. [35] and, seed sphericity (Ø) and volume (V), according to Mohsenin [36]:

$$R_a = \frac{b}{a}100 \quad (5)$$

$$\varnothing = \left[\frac{(abc)^{1/3}}{a}\right]100 \quad (6)$$

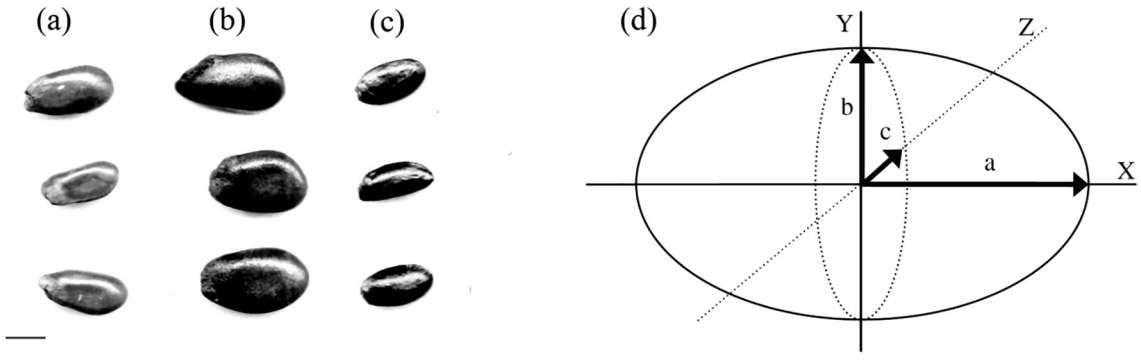

Fig. 1 – Seeds of (a) *A. coriacea*, (b) *A. montana*, and (c) *A. squamosa*. Characteristics of the three dimensional geometry (d) of seeds: a- length; b- width; c- thick. Bars=10mm.

Table – 1. Species, taxonomic position, number of plants analyzed and herbarium voucher.

Species	Number of individuals	Number of herbarium voucher	Coordinates
Order: Magnoliales			
Family: Annonaceae			
A. coriacea Mart.	12	DDMS 5419	22°05′48,2″S and 55°15′55,1″W
A. montana Macfad.	12	DDMS 5681	22°05′48,2″S and 55°15′55,1″W
A. squamosa (L.) Bark.	15	DDMS 5101	21°33′51.29″S and 42°26′58.44″W

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$$V = \pi \frac{abc}{6} \quad (7)$$

where a, b, and c represent the length, width, and thickness of the seeds, respectively.

2.3. Elliptic Fourier descriptors

Image analysis using elliptic Fourier descriptors (EFDs) was used in order to evaluate shape variation in seeds [37]. Fifty seeds were sampled for each species simultaneously scanned at 300 dpi resolution using a digital image scanner (HP Deskjet F4 100 flatbed scanner, Hewlett–Packard, USA). We saved each image in red–green–blue (RGB) color format (BMP) with 256 levels (*i.e.*, 8-bit resolution per channel). Using SHAPE (version 1.3), the seed contours were extracted from the images and delineated according to the EFDs. The shape variations captured by the EFDs were decomposed into statistically independent characteristics by the principal component analysis (PCA) of EFDs. The PCA was performed on the variance-covariance matrix of the EFDs.

To calculate EDDs, the contour of the digitized shapes is represented as a sequence of the x- and y- coordinates of ordered points measured contour-clockwise from an arbitrary starting point. The elliptic Fourier expansion of the sequences of the x-coordinates can be calculated as following equation:

$$xp = xcen + \sum_{n=1}^{\infty} (a_{cos} = a_n \cos)\left(\frac{2n\pi t_p}{T} + b_n sen \frac{2n\pi t_p}{T}\right) \quad (8)$$

where, T is the perimeter of the contour ($T = t_k$) and k is the total number of the points on the contour. The x-coordinate of the p^{th} point is x_p ($x_p = \sum_{i=1}^{p} \Delta x_i$), Δx_i is the displacement along the x-axis of the contour between the $(I-1)^{th}$ and i^{th} points. x_{cen} is the coordinate of the center point, and n is the harmonic number of the coefficients (a_n and b_n). For the y-coordinates coefficient values (c_n and d_n) data are found in the same way.

2.4. Optical characteristics of seeds

The light response curves of ChlF were obtained by measuring using a portable fluorometer FluorPen FP-100 PSI (Photon Systems Instruments, Czech Republic). Fifty seeds were sampled for each species. Seeds were dark-adapted for 30 min and submitted to 1 s upon 1.500 μmol m^{-2} s^{-1} light pulse. The data were processed and analyzed with FluorPen (version 1.0.4.2). ChlF induction curve obtained were pre-processed prior to computational analysis. A fast Fourier transform (FFT) and Short-Time Fourier transform (STFT) was performed to access real and imaginary spectrum and time-frequency domain spectrogram.

2.5. Data analysis

Data on relative structural traits were expressed as mean and range (maximum and minimum) in order to determine the possible association between phenotypic variation and the analyzed variables. Normality was evaluated by Kolmogorov-Smirnov test. Outliers were removed. The Student's *t*-test pos-hoc test was used to analyze the associations between species and the variables. The Spearman's correlation test was used for obtaining the Rho values. Statistical analyses were performed using the R software [38]. Probability level (p-value) <0.05 was assumed.

3. Results

3.1. Structural characteristics of seeds

The values of linear axis and geometric dimensions of seeds are presented in Table 2. Significant differences ($p < 0.05$) among the dimensional patterns of seeds were observed. Seed biometry revealed mean value of the length, width, and thickness of 18.47, 11.07 and 6.64 mm; 15.50; 9.12 and 5.64 mm; and 12.86; 6.78 and 4.77 mm for seeds of *A. coriacea*, *A. montana* and *A. squamosa*, respectively. Variations estimated on diameters (Table 2) were observed for D_a and D_g - between 12.05 and 11.03 mm in *A. coriacea*, 10.09 and 9.24 mm for *A. montana* and 8.14 and 7.43 mm in *A. squamosa*. The highest value of coefficient of variation (CV) was observed in thickness of *A. coriacea* (10.35%), whereas, a minor value of D_e was noted in *A. squamosa* (3.55%).

Based on values of kurtosis and asymmetry, a positive asymmetry was observed for *A. coriacea* in length and D_a, for *A. montana* in width and thickness and for *A. squamosa* in thickness. All other axial and geometric variables showed negative asymmetry. However, total amplitude (Λ) revealed a dimensional homogeneity higher for *A. montana* than the *A. coriacea* and *A. squamosa* seeds. All observed variables in seeds of *A. coriacea* (Table 2) showed high standard deviation (SD > 1).

Seed surface area (S_A) and volume (V) were 368.73 mm^2 and 728.38 mm^3 (*A. coriacea*), 268.57 mm^2 and 414.76 mm^3 (*A. montana*) and, 174.29 mm^2 and 217.22 mm^3 (*A. squamosa*). Based on S_A and V, significant differences ($p < 0.001$) between seeds were observed, as shown in Fig. 2.

Based on their values of coefficient of determination ($r^2 >$ 99%) between seeds S_A and V, a high positive trend for linearity was observed (Fig. 3).

These seeds presented three unequal semi-axes, and could be described as being scalene ellipsoidal in shape. The values of Ø are distant from 1.0 (100%) which indicates a sphere, observed values were lower than 35% ($p < 0.001$). As for the aspect ratio (R_a), only *A. squamosa* differed ($p < 0.001$) from the other two species (Fig. 4), where *A. coriacea* seeds showed positive asymmetry for aspect ratio.

The results showed significant correlations among dimensional traits evaluated (Table 3), both at 5% and 1% of significance, where it was observed that most of correlations showed a value $p < 0.001$ for the three species. The highest positive correlation (Rho = 1.000, $p < 0.001$) was observed between S_A and D_a for all the valuated seed species. Very high positive correlation was observed for (Rho = 1.000, $p < 0.001$) between V and S_a and between V and D_a in *A. montana* and *A. squamosa*. Negative correlations were found as well, *i.e.*, Rho = −0.392, $p < 0.05\%$ between R_a and thickness for *A. coriacea*. *A. montana* showed the highest negative correlation (Rho = −0.892, $p < 0.001$) between R_a and length and finally (Rho = −0.049, p < 0,001) between R_a and D_g for seeds of *A. squamosa*.

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Table 2 – Estimated values of the evaluated parameters and significance of the differences analyzed by Student's t test among seeds of three species of the genus *Annona* (Annonaceae). [a=length (mm); b=width (mm); c=thickness (mm); D_a=geometric mean diameter (mm); D_e=equivalent mean diameter (mm); D_a=arithimetic mean diameter (mm); CV=coefficient of variation; Min=minimum value; Max=maximum value; $\bar{x}$ = mean; σ^2 = variance; *sd*=standard deviation; se=standard error; S=assimetry; K=kurtosis; = total amplitude; p=level of significance by the Student t test].

	a	*b*	*c*	D_a	D_g	D_e
Seed species: A. coriacea Mart.						
Min	13.65	8.55	3.78	9.11	8.26	8.52
$\bar{x}$	18.47	11.07	6.64	12.05	11.03	11.28
Max	22.91	13.31	8.29	14.54	13.19	13.49
σ^2	4.89	1.49	1.02	1.54	1.37	1.36
Sd	2.21	1.22	1.01	1.24	1.17	1.17
Se	0.22	0.12	0.10	0.12	0.12	0.12
CV(%)	11.98	11.01	15.24	10.31	10.61	10.35
S	0.08	−0.18	−0.48	0.01	−0.21	−0.15
K	−1.01	−0.74	−0.44	−0.92	−0.78	−0.83
Λ	9.26	4.76	4.51	5.43	4.91	4.97
p-value	ns	ns	ns	ns	ns	ns
Seed species: A. montana Macfad						
Min	11.62	7.41	4.08	8.77	8.42	8.56
$\bar{x}$	15.50	9.12	5.64	10.09	9.24	9.43
Max	17.94	11.17	7.06	10.9	9.95	10.12
σ^2	2.08	0.50	0.36	0.17	0.12	0.11
Sd	1.44	0.71	0.60	0.41	0.35	0.33
Se	0.14	0.07	0.06	0.04	0.03	0.03
CV(%)	9.31	7.79	10.72	4.08	3.81	3.55
S	−0.56	0.44	0.30	−0.86	−0.09	−0.29
K	−0.13	0.59	0.16	1.18	−0.48	−0.32
Λ	6.32	3.76	2.98	2.12	1.53	1.55
p-value	**	ns	**	ns	ns	ns
Seed species: A.squamosa (L.) Bark.						
Min	6.90	5.20	3.80	6.20	6.05	6.18
$\bar{x}$	12.86	6.78	4.77	8.14	7.43	7.52
Max	15.90	8.20	7.10	9.03	8.19	8.27
σ^2	1.90	0.30	0.32	0.22	0.15	0.14
Sd	1.38	0.55	0.56	0.47	0.39	0.38
Se	0.14	0.05	0.05	0.05	0.04	0.04
CV(%)	10.72	8.18	11.79	5.22	5.02	5.82
S	−1.24	−0.07	0.85	−1.27	−0.82	−0.99
K	3.23	−0.23	1.74	3.37	1.59	2.10
Λ	9.00	2.90	3.30	2.83	2.14	2.08
p-value	**	**	**	*	*	ns

ns: did not significantly differ from each other, *: indicate statistic difference between variables ($p < 0.05$), **: indicate statistic difference between variables ($p < 0.001$).

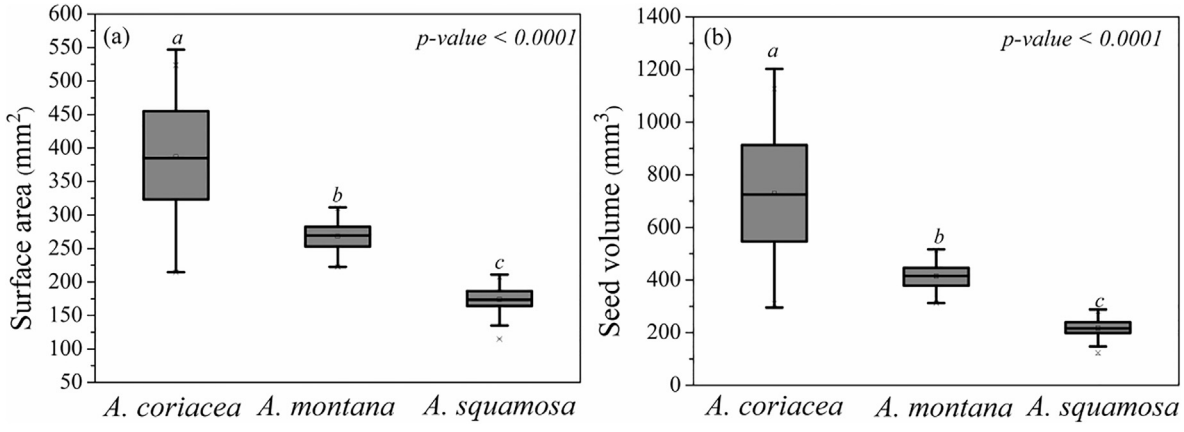

Fig. 2 – Boxplot of surface area (A) and volume (B) among seeds of three species of the genus *Annona* (Annonaceae).

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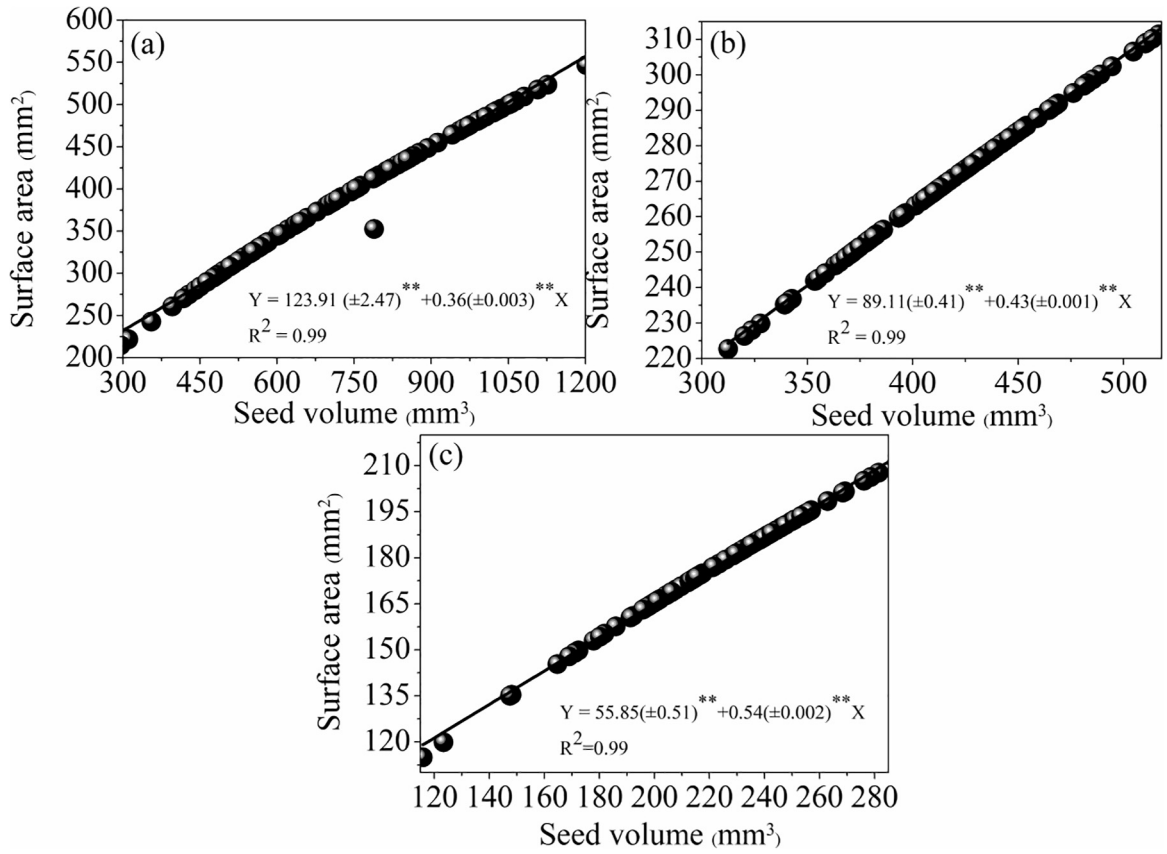

Fig. 3 – Linear fit-regression of surface area and volume among seeds of three species of the genus *Annona* (Annonaceae). (a) *A. coriacea*, (b) *A. montana*, and (c) *A. squamosa*. p<0.001.**

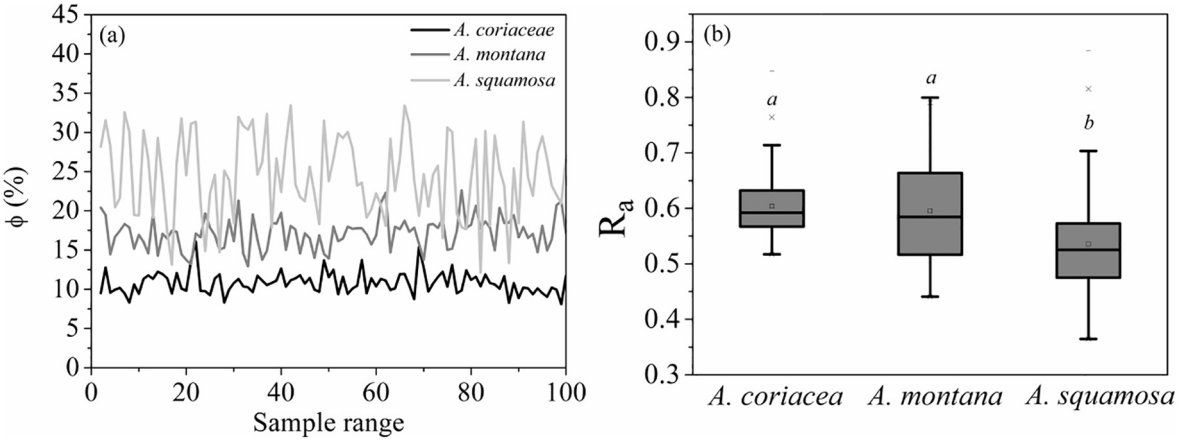

Fig. 4 – Projected oscillation on the sphericity (a) and boxplot of aspect ratio (a) among seeds of three species of the genus *Annona* (Annonaceae).

3.2. *Shape variation based on elliptic Fourier descriptors*

The PCA results of seed shape are displayed in Fig. 5. The coefficients of the elliptical Fourier descriptors were recalculated inversely using a matrix Eigen-vector, allowing the marker as the main component, and defined to be equal to the mean ± 2 S.D., while scores remained in other components relative to the mean. Fig. 5 shows variations of PCs from the first to fifth score between the 3 species of *Annona* ssp.

In the principal components analysis, scores PCA 1 and PCA 2 (based on the shape of the seeds) for the 3 species of the genus *Annona* were represented in the dispersion diagram (Fig. 6). The mean values estimated for the PCA 1 and PCA 2-varied at 1.9×10^{-7} and 2.3×10^{-7} in *A. coriacea*, 4×10^{7} and 3.2×10^{-7} in A. *montana* and 1.5×10^{-5} and 3.5×10^{-6} in A. *squamosa* (Fig.S1b–c), respectively. The frequency distributions (Fig. S1) clearly reflect the differences in variance and the mean of the shape of the seed among the studied species.

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Table 3 – Array of the Spearman correlation coefficients for biometric and physical variables among seeds of three species of the genus *Annona* (Annonaceae). [a=length (mm); b=width (mm); c=thickness (mm); D_a=geometric mean diameter (mm); D_e= equivalent mean diameter (mm); D_a=arithimetic mean diameter (mm); S_A=surface area (mm^3); Ø = sphericity (%); R_a=aspect ratio (a.u.); V=volume (mm^2)].

A. coriaceae Mart.

	a	*b*	*c*	D_a	D_g	D_e	S_A	Ø	R_a	V
A	1.0000									
B	0.7545	1.0000								
C	0.6015	0.3224	1.0000							
D_a	0.9073	0.7752	0.7878	1.0000						
D_g	0.9117	0.8453	0.7039	0.9847	1.0000					
D_e	0.9234	0.8147	0.6465	0.9608	0.9766	1.0000				
S_a	0.9073	0.7752	0.7878	1.0000	0.9847	0.9608	1.0000			
Ø	0.8274	0.7469	0.8544	0.9669	0.9443	0.8900	0.9669	1.0000		
R_a	-0.3372	0.2844	-0.3928	-0.2543	-0.1621	-0.2348	-0.2543	-0.1411	1.0000	
V	0.8997	0.7655	0.7977	0.9975	0.9808	0.9567	0.9975	0.9657	-0.2633	1.0000

A. montana Macfad.

	a	*b*	*c*	D_a	D_g	D_e	S_A	Ø	R_a	V
A	1.0000									
B	-0.5044	1.0000								
C	-0.2569	-0.0884	1.0000							
D_a	0.2303	0.2108	0.6070	1.0000						
D_g	-0.1834	0.4287	0.4045	0.9481	1.0000					
D_e	0.7250	-0.0374	0.1596	0.7899	0.7806	1.0000				
S_a	0.2303	0.2108	0.6070	1.0000	0.9481	0.7899	1.0000			
Ø	-0.5430	0.5527	0.7247	0.6546	0.6453	0.1186	0.6546	1.0000		
R_a	-0.8923	0.8081	0.1130	-0.0593	0.0827	-0.4751	-0.0593	0.6228	1.0000	
V	0.2303	0.2108	0.6070	1.0000	0.9481	0.7899	1.0000	0.6546	0.05993	1.0000

A. squamosa (L.) Bark.

	a	*b*	*c*	D_a	D_g	D_e	S_A	Ø	R_a	V
A	1.0000									
B	-0.2211	1.0000								
C	0.0419	-0.2401	1.0000							
D_a	0.4968	0.2391	0.6420	1.0000						
D_g	0.4869	0.3724	0.5195	0.9774	1.0000					
D_e	0.8347	0.1039	0.3428	0.8658	0.8711	1.0000				
S_a	0.4968	0.2391	0.6420	1.0000	0.9774	0.8658	1.0000			
Ø	-0.136	0.4294	0.7432	0.7461	0.7280	0.3672	0.7461	1.0000		
R_a	0.8156	0.6875	0.1139	-0.1852	-0.1063	-0.4948	-0.1852	0.3752	1.0000	
V	0.4968	0.2391	0.6420	1.0000	0.9774	0.8658	1.0000	0.7461	-0.1852	1.0000

[ ] Rho = 1.000; [ ] level of significance 1%; [ ] level of significance 5%; [ ] non-significantly by the Student t test.

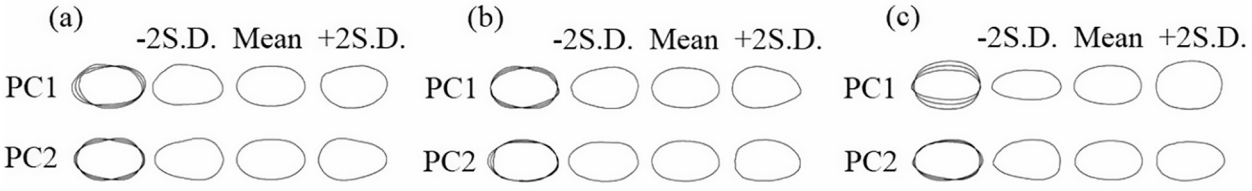

Fig. 5 – Visualized shape variation in leaf contour images by the five most influential principal components. PC1 to PC2 correspond to the first to second principal components, respectively. From the right, each column shows the case where the score takes +2 SD (standard deviations), mean and -2 SD based on coefficients of Elliptic Fourier Descriptors among seeds of three species of the genus *Annona* (Annonaceae). (a) *A. coriacea*, (b) *A. montana*, and (c) *A. squamosa*.

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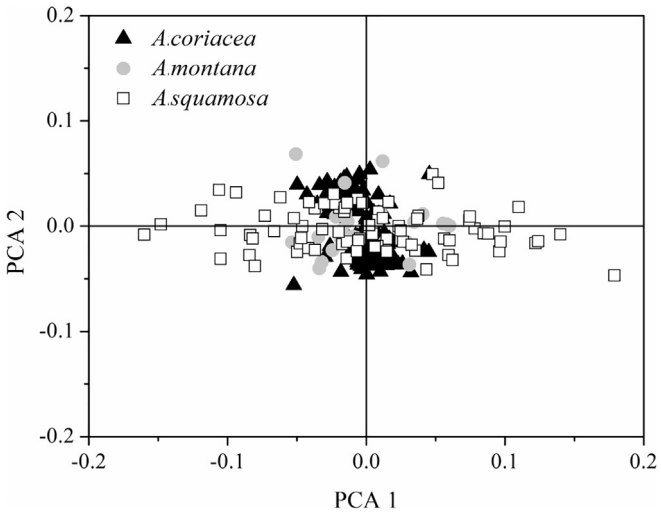

Fig. 6 – Scatter diagram of the first and second principal components (PCA 1 and PCA2) based on coefficients of Elliptic Fourier Descriptors among seeds of three species of the genus *Annona* (Annonaceae).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.inpa.2018.07.001.

3.3. *Optical characteristics of seeds*

In Fig. 7(a)–(c) comparing the kinetics of ChlF of seeds in the studied species showed differences in the intensity and

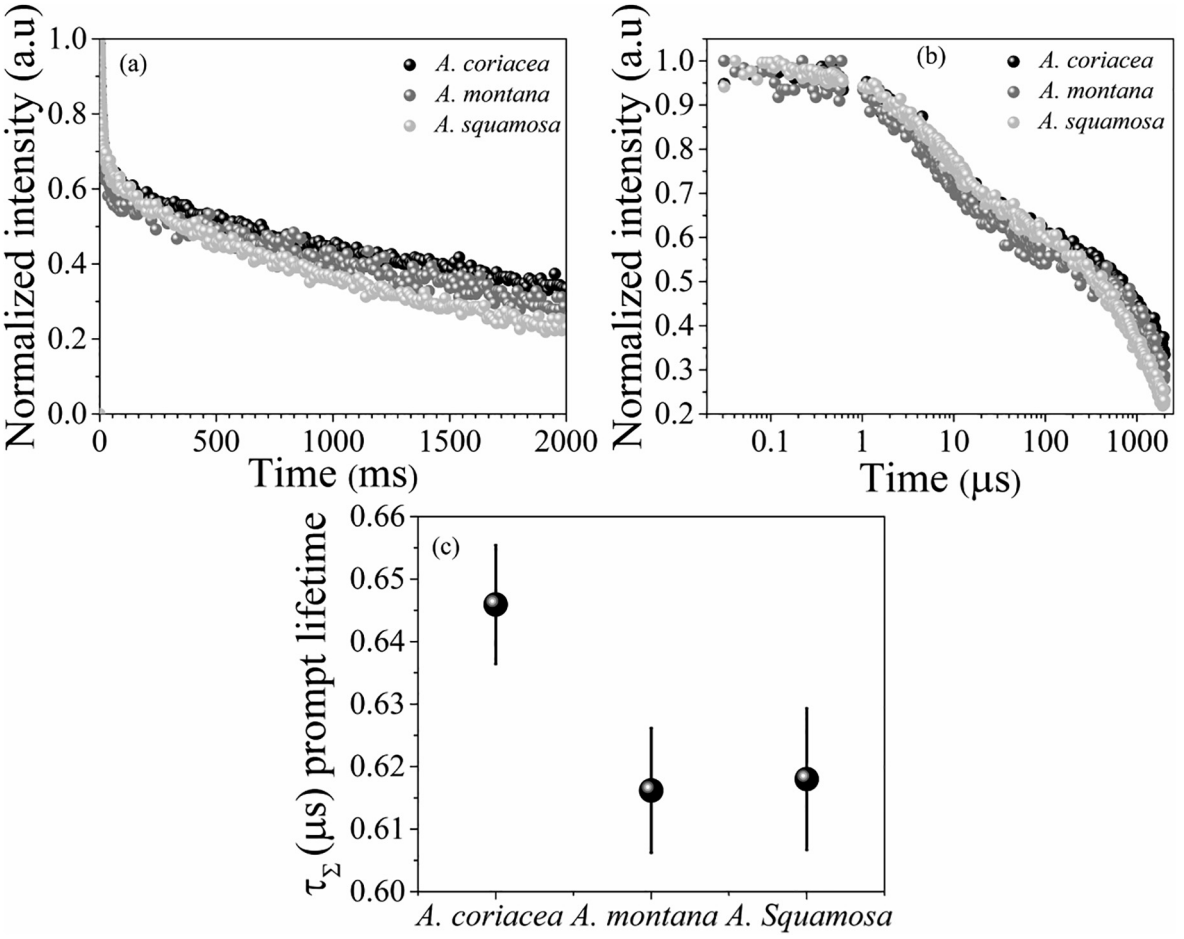

Fig. 7 – Characteristics of fluorescence induction curves of tegument among seeds of three species of the genus *Annona* (Annonaceae). (a) Fluorescence decay at ms scale; (b) Fluorescence decay at s scale; and (c) fluorescence lifetime.

oscillatory behavior over time. Highest fluorescence intensity was observed for *A. squamosa*, whereas a reduced intensity was observed for *A. montana*. All species presented fast fluorescence decay as well.

Fig. 8(a) showed the patterns of real spectrum of ChlF measured in the seeds, while Fig. 8(b) shows the patterns ofi maginary spectra. The real and imaginary spectra (first 10 Hz frequency) have different shapes. The real spectrum is always positive and decreases with frequency, whereas the imaginary spectrum present negative values which increase over frequency. Both the spectra allowed discrimination among the seeds, where the real spectrum and imaginary spectra showed positive and negative magnitudes, respectively, with a greater difference of peak height between the species at 1 Hz frequency. Our results demonstrate visual differences among all species studied in real and imaginary spectra.

Aiming to unraveling the fluorescence behavior, the variation observed for ChlF over time (Fig. 7a–c) and in the frequency domain (Fig. 8), a STFT analysis of the ChlF was conducted as follows. Their differences were reflected in the2D map obtained by means of STFT (Fig. 9). In general, each spectrum of ChlF represents the intensity of the emitted light by a seed (scale: 0-dark blue to 100-red) in a time-frequency representation. The colors show the relative magnitude or energy level of the signal at different times (0 to 1000 ms) and frequency. The samples of *A. squamosa* showed high complexity in the two-dimensional spectrum, displaying the largest peaks of ChlF amplitude detectable, in accordance with observed data for the morphologic parameters – *i.e.*, S_a, V, Ø and R_a values.

4. Discussion

Generally speaking, analysis of the total amplitude (Λ) of samples on seed dimensions reflected a higher dimensional homogeneity pattern in seeds of *A. montana*, and a higher dimensional heterogeneity for the seeds of *A. coriacea* (Table 2). Comparing observed results in this study for the linear dimensions of the orthogonal axes in seeds, with data from Lima-Brito et al. [39] and Pimenta et al. [40] in *A. muriatica* and *A. crassiflora*, we noted that our morphological data remain to be corroborated by patterns in seeds of *Annona* species. Overall, the assessed seeds presented three unequal

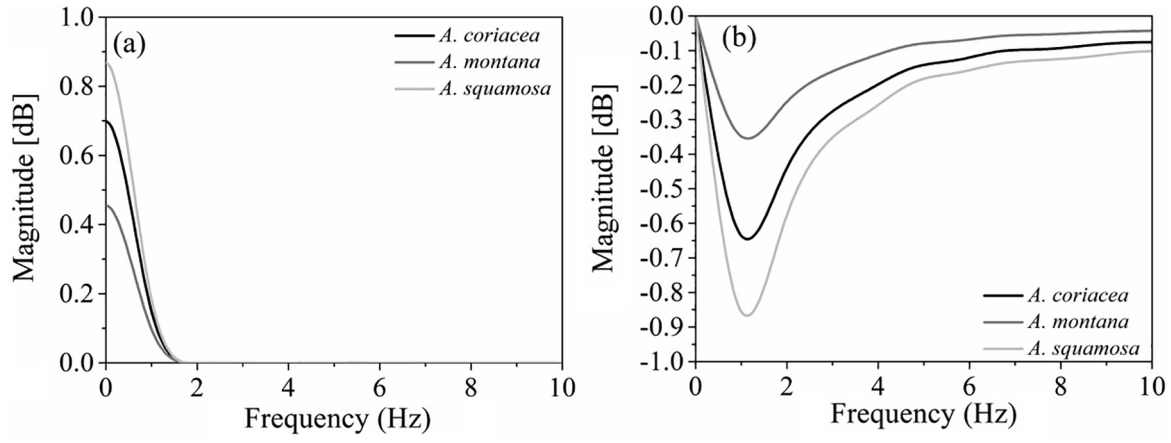

Fig. 8 – Real (a) and imaginary spectrum (b) for the first 10Hz among seeds of three species of the genus *Annona* (Annonaceae) by applying Fast Fourier transform on the fluorescence induction curves.

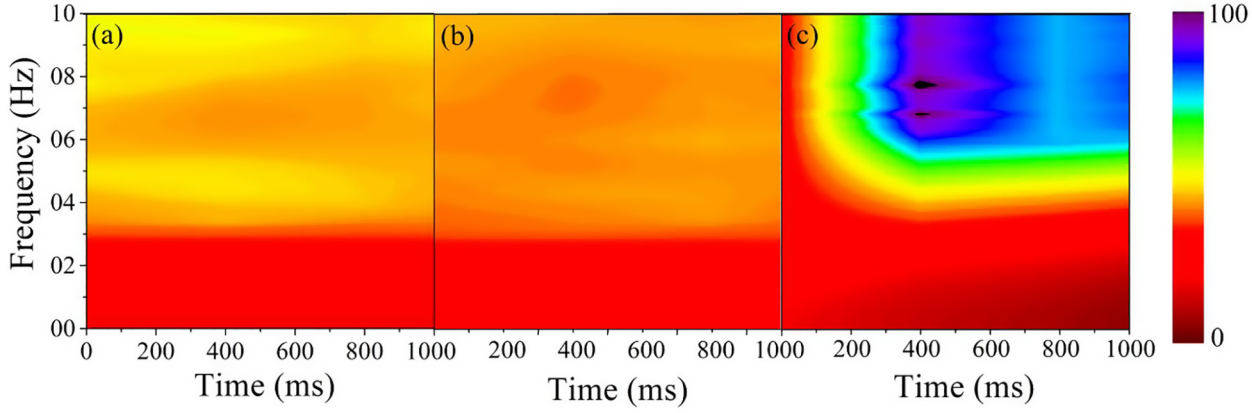

Fig. 9 – The average of time-frequency domain. Two-dimensional counts map per bin based on short-time Fourier transform data of fluorescence induction curves among seeds of three species of the genus *Annona* (Annonaceae). (a) *A. coriacea*, (b) *A. montana*, and (c) *A. squamosa*.

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semi-axes, associated with low values of the Ø and R_a (Fig. 3), suggesting a dimensional classification for these species, such as ellipsoid scalene, similar results were obtained in Chia seeds [11].

According to Mohsenin [36], the seeds volume is defined based on occupation of an object in three-dimensional space; solids volume depends on a number of factors of its linear dimensions, surface area, weight, and temperature. Very high positive correlation was observed for the studied species (Rho = 1.000, $p < 0.001$) between V and S_a and between V and D_a - in *A. montana* and *A. squamosa*, and the highest positive correlation (Rho = 1.000, $p < 0.001$) between S_a and D_a for all evaluated species. Thus, we provide corroborating evidence on the relationship between V and S_A (Fig. 2), the coefficient of determination (R2) which showed a high value (>99%). A high negative correlation (Rho = −0.983, $p < 0.001$) was only observed between length and $R_a$in *A. montana*, thus providing evidence that the linear dimensions affect positive or negatively dimensional structure of the measured seeds.

Although the genetic differences are relatively important, the seed physical and/or structural patterns may be influenced by factors such as fruit maturation stage [41,42], and abiotic or biotic stress stimulus, including climate change and the stimulus of interactions inter and intraspecific upland during seeds development.

Seed pre-processing is an important step in seedling production. Some structural patterns are critical for separation of viable seeds from non-viable seeds and inert materials based on physical characteristics alone using gravity tables [13,15,43] whereas the higher dimensional uniformity is reflected easily in the separation of seeds from the residual material (fruits, leaves, and twigs), thus minimizing time and costs in processing stages. For the seeds of native species, the lower dimensional uniformity due to the genetic variability [7] is a challenge into mechanization and automation process [44] which can be more challenging in high demand facilities for seed production.

The dimensional relationship of seeds also influence storage, by affecting the drying time and space required for packaging because of its volume [45], in addition to effects on the water uptake during seed hydrolysis suggesting the impact of these variables (specifically, volume and surface area) on germination [46]. Seed shape are linked consistently to a longevity of seeds in the soil seed bank [47], explaining fire or heat tolerance of seeds derived from burning [48]. Directional selection act arising from the preference to disperser in the selection of fruits and seeds [49–51].

In a physiological study conducted on physical properties of pearl millet seeds, the author observed an increase for both, volume and surface area, as a function of the increase of water content in seeds [52]. The results showed that relationships between surface area and volume of these seeds lifts up the need for more physiological studies based on these characters not only in species of Annonaceae but also in other native woody species. Due to the fact that seed size are one of the most important aspects of plant [2,7,53].

Recorded variables for the three *Annona* species presented in this study contribute to their biological knowledge and provide benefits for pre-processing and processing of seeds in agricultural systems, additionally contributing with data for future taxonomic studies, emphasis on structural characteristics which are uniform in terms of taxon, genus and/or family, or even to differentiate seed lots

Size and shape of the seeds are genetically determined, but may undergo changes during its development by environmental factors [54]. Nevertheless, dimensional patterns observed for the seeds of all three studied species allowed comparative studies with other species of the same genus, in addition to assessing changes in interspecific morphology of seeds among species in the same genus and in populations located in different geographic regions, or in different environmental conditions. We detected the quantitative changes occurring in seed shape traits based on EFD. As mentioned above, interspecific distinction based on the variations in the seed shape are explained by means PC scores (PC 1 and PC 2) of EFD, in which the PC1 highlights these variations, with an emphasis on the greater dispersion dimension to *A. squamosa*.

Although the dimensional variables provide useful information on the characterization of the species, when combined with other tools can provide more robust information about distinctions between the sampled material, as for example, separation of varieties through analysis of fluorescence emission spectra [21,51–57] in which its potential has not been fully explored yet by seed scientist and technologists.

The variations in ChlF emission showed a lower intensity in *A. montana,* and higher in *A. squamosa* (Fig. 8), which also, visibly showed increased separation from the other species in real and imaginary spectra (Fig. 9). The results of ChlF observed in three species of *Annona* studied may represent intraspecific differences upland imperceptible when observed in the visible spectrum (VIS), whereas the higher absorption of energy associated with the dark color of the seed tegument due to anthocyanins concentration [58–60], so that emissions in the red spectral region, characteristic of the chlorophylls, can contribute to species selection. Moreover, variations in the intensity of ChlF emission can be associated with natural loss of vigor, maturation stage of the embryo or even during the process of seed imbibition, thus varying in different stage before and after metabolic activation [25,61,62] to involve both pre-existing chlorophyll as those which will be synthesized when the conditions (light precursors) are favorable [5].

Changes in ChlF emission can even be associated with biotic or abiotic stresses interfering in the relationships of energy absorption/dissipation, affecting elements of the light-harvesting complex (LHC) and other molecules associated with the electron transport chain, in the case of manifest spreading across the spectrum [63] and through the fluorophores production that may affect the number, location and intensity of fluorescence peak [64]. Although temporal dynamics has allowed to compare both oscillatory variations and distinctions into intensity decay of ChlF among the three species (Fig. 7a–c), the magnitude of frequency, in particular that of the imaginary spectrum (Fig. 8b), as well as time/frequency representation displayed in a two-dimensional map (Fig. 9) were more efficient in species-level discrimination.

The analysis of ChlF in the frequency domain by means of FT has been described as a promising tool in plant physiological status evaluation in response to different concentrations

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of herbicide stressors [65]. We observed a relative uniformity in the intensity of time-resolved fluorescence for all the three species (Fig. 7a–c), including the relatedness in frequency spectra (Figs. 8 and 9). It is reasonable to say that the expression of similarities and differences of energy dissipation behavior at a given time, considering its seed physiology and the nature of this fluorescent material is based on the supramolecular fluorophore complex. Under these experimental conditions, where the fluorescence output depends on the physiological stage (vigor, maturation stage of embryo, among others) out study falls short of explaining these, as they were not the objectives of the presented study. On the other hand, a lack of ChlF data in literature about the three species did not allow any comparison of the data. However, our results agrees with current findings in literature, for example, Qiu and co-workers [66] differentiate the freshness of leafy vegetables at different postharvest time using variables extracted from fluorescence induction curve.

5. Conclusions

Comparison between three *Annona* (*A. coriacea*, *A. montana* and *A. squamosa*), presented in this study contribute to their biological knowledge. Dimensional variables recorded for seeds provide benefits for pre-processing and processing of seeds in agricultural systems, additionally contributing with data for future taxonomic studies, combined to ChlF technique, seems promising to the taxon differentiation specially if applied in later studies of stress in seeds. This study indicates that the combination of seed shape traits and time-resolved ChlF emission signal is a very appropriate methodology to be used in seed scale for agronomic and taxonomic studies. A more detailed germinative study of this correlation, together with agronomic traits relationships, will be the subject of future research.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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