

Morphological and Physiological Germination Aspects of *Anadenanthera colubrina* (Vell.) Brenan

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Abstract

Anadenanthera colubrina is a species native to Brazil, from the Fabaceae family and has potential for use in the timber industry, in the reforestation of degraded areas, besides having medicinal properties. Its propagation is mainly by seeds, but basic subsidies regarding the requirements for optimal germination conditions are still lacking. Aiming to contribute to the expansion of its cultivation, rational use and conservation, the objective of this study was to investigate the morphology and anatomy of fruits and seeds, as well as the responses to factors as thermal regimes and substrates in seed germination. The 1000-seed weight and seeds per fruit were determined. To characterize the seed tissues, histochemical test with Sudan III and Lugol was used. The temperatures analyzed in the germination test were 15-25 °C; 25 °C; 20-30 °C and 30 °C. Different substrates for germination (paper rolls, paper sheet such as "germitest", commercial substrate and sand) were also analyzed in the presence of light at 30 °C. The average number of seeds per fruit is 10 and the 1000-seed weight is 118 g. Germination is fast and high over a wide temperature range; however, the temperature of 25 °C contributes to a substantial increase in the percentage of abnormal seedlings and dead seeds, both in the absence and presence of light. *A. colubrina* seeds are indifferent to light and the highest vigor was verified at 30 °C. Sand and commercial substrates are efficient in initial seedling development.

Keywords: 'Angico-vermelho'; seedling generation; substrate; temperature

Introduction

In recent years, there has been a growing interest in the propagation of native forestry species, emphasizing the need for recovery of degraded areas and landscape restoration (Guerra and Jorge, 2017). In this context, studies involving seed technology, species ecophysiology and regeneration dynamics, associated with floristic and phytosociological surveys, are extremely important for the establishment of models that can be adopted and applied in management programs and to conduct forest regeneration processes (Candiani, 2006; Miranda *et al.*, 2017). Thus, it is necessary to obtain basic information about the germination, cultivation and potentiality of native species, aiming at their use for the most diverse purposes. However, the available

knowledge is still incipient when considering aspects such as seed analysis and management of most potential species, in order to provide data that can characterize their physical and physiological attributes (Ribeiro-Oliveira and Ranal, 2014).

Among the prominent native forest species from Brazil with potential for restoration of degraded areas is *Anadenanthera colubrina* (Vellozo) Brenan var. *cebil* (Griseb.) Altschul, belonging to the *Fabaceae* family (Pires *et al.*, 2016). *A. colubrina* shows natural regeneration by seeds and has annual production, with barochory dispersal (Carvalho, 2003).

However, the morphological diversity within the *Fabaceae* family generates a series of taxonomic problems, increasing the search for information about the fruits, seeds and seedlings from this family species in order to complement those from vegetative organs and flowers

(Oliveira, 1999). Therefore, the biometric characterization of fruits and seeds, as well as the study on seed requirements regarding the effects of temperature, light and ideal substrate for germination, provides important subsidies for the differentiation of pioneer and non-pioneer species in tropical forests and species from the same genus related to the characteristics of seedling dispersal and establishment, especially for species typical of secondary vegetation (Cruz *et al.*, 2001; Fenner, 1993; Silva and Filho, 2006; Carvalho and Nakagawa, 2012).

Therefore, the objective of this study was to analyze the morphology and anatomy of fruit and seeds, the chemical composition of seeds, as well as their response to factors as imbibition, thermal regimes and substrates in germination.

Materials and Methods

Plant material

A. colubrina fruits were collected in approximately 50 matrix trees located at Universidade Federal de Lavras (UFLA), Lavras - Minas Gerais, Brazil, at the geographical coordinates 21°13'17"W and 44°57'47"W. After collected, the fruits were processed and the seeds were removed and divided into two seed lots (A: Seeds that were naturally dispersed, i.e., collected on the ground; B: Seeds obtained from closed fruits). The seeds were packed in polyethylene packages and stored in a cold chamber (8 °C with 45% relative humidity), as recommended by Espinoza (2004).

Morphological characterization of fruits and seeds

Seeds (lots A and B) were mechanically ground by the oven dry method for 24 hours at 105 ± 3 °C with values expressed in percentage on a wet basis (Brasil, 2009). Ripe fruits of brown color were randomly selected (approximately 100) from 50 matrix trees. A calliper with a precision of 0.05 mm was used to measure the length and width of fruits and seeds. Length was defined as the longitudinal distance between the apex and the base, whereas width corresponded to the measurement perpendicular to the median region of the fruits, expressed in centimeters (cm). The number of seeds per fruit was obtained from a sample consisting of 100 fruits and the 1000-seed weight was determined using eight replicates of 100 seeds, weighed in an analytical balance with a precision of 0.001 g, according to Brasil (2009); data average was expressed in grams (g).

Histochemical tests

The seeds were submitted to manual cross sections and subsequent staining with safranin (Kraus and Arduin, 1997) and phloroglucinol (Foster, 1949). The cotyledons and embryonic axes were cross-sectioned to determine the type of reserve using the following reagents: Lugol, to qualify the presence of starch, and Sudan IV, for the identification of lipid bodies (Kraus and Arduin, 1997). The assembly of semi-permanent slides followed the techniques described by Johansen (1940). Visual observations were performed using an Olympus CBB microscope with photographic documentation using a digital camera (Canon®), with resolution of 7.0 megapixels and 8× optical zoom.

Ultrastructural characterization of embryonic axes

For scanning electron microscopy, embryonic axes were collected and immersed in fixative solution (Karnovsky) pH 7.2 for a period of 24 hours, washed in sodium cacodylate buffer three times and post-fixed in 1% osmium tetroxide for 1 hour at room temperature. After this period, they were washed three times in distilled water and then dehydrated in increasing acetone gradients for 10 minutes each (25%, 50%, 75%, 90% and 100% three times). Subsequently, the material was taken to the critical point apparatus for complete drying, mounted on stubs and covered with gold. The specimens were observed in a LEO Evo 40 Scanning Electron Microscope, according to methodology described by Alves (2004). Ten replicates were used per treatment.

Effect of temperature and luminosity on seed germination

Seeds from lots A and B were submitted to different thermal regimes, in the absence or presence of constant light in an environment with 100% relative humidity. Germination tests were conducted at alternating and constant temperatures (15-25 °C; 25 °C; 20-30 °C and 30 °C), in a BOD germination chamber with photoperiod control. The substrate used was paper roll, moistened with distilled water 2.5 times the paper weight, placed in 250 mL beakers containing 2.0 cm water and covered with plastic bags to standardize humidity. In order to obtain the absence of light, the beakers were involved in black polyethylene packages. Germination was evaluated using a radicle protrusion of ± 2.0 mm. Germinated seeds were kept in the paper roll in order to follow the initial growth and normal seedling count; evaluations were then performed after ten days. Abnormal seedlings were considered as having darkened apex root or epicotyl, absence of epicotyl or root, atrophied root or shoot at the end of the experiment. The percentage of dead seeds was also evaluated, having as criteria tissue softening and the presence of mycelium. Germination Speed Index (GSI) was determined together with germination and calculated according to the equation proposed by Maguire (1962). The treatments were distributed using a completely randomized design (CRD) and the analysis of variance was performed in a $2 \times 2 \times 4$ factorial design (seed lots \times luminosity conditions \times temperatures), with four replicates of 25 seeds for each treatment. Percentage data of normal and abnormal seedlings were transformed using \sqrt{x} , aiming at meeting the presumptions from the analysis of variance. For the other variables, there was no need for transformation.

Effect of substrate on seed germination

Seeds from lots A and B were submitted to different substrates for germination, paper roll, paper sheet (such as "germitest"), commercial substrate and sand, in the presence of light at a constant temperature of 30 °C. Paper sheet was moistened with an amount of distilled water equivalent to 2.5 times the dry paper weight; for the other substrates, acrylic boxes (gerbox) were used; water was added as necessary. A germination chamber was used, maintaining 100% relative humidity (Brasil, 2009). Germination was evaluated daily, using a radicle protrusion of ± 2.0 mm as a criterion. At the end of the experiment, after 10 days, the percentage of normal and abnormal seedlings, besides dead

seeds, was evaluated. GSI was analyzed together with the germination test; the evaluations were performed daily from the radicle protrusion and calculated according to the equation proposed by Maguire (1962). The healthy test was performed, evaluating the presence of fungi in the seeds (qualitative data), using a magnifying glass and stereoscopic microscope due to their appearance.

Morphological characterization of seedlings in different substrates

The seedlings obtained in the previous test were evaluated after 10 days for shoot and root length, measured from 15 normal seedlings of each replication, with the aid of a graduated scale in millimeters (mm). In order to determine the dry matter of the shoot and root system of normal seedlings, the lots were separated and placed in paper bags previously identified and taken to a convection oven regulated at 60 °C for 72 hours. After this period, the seedlings were removed from the oven and weighed in an analytical balance with a precision of 0.001 g; the results were expressed in grams (g). The treatments were distributed according to the CRD and the analysis of variance was performed in a 4 × 2 factorial design (substrates × seed lots), with four replicates of 25 seeds for each treatment.

Statistical analyses

The obtained data were subjected to analysis of variance using the Sisvar statistical software (Ferreira, 2014). The average among treatments was compared by Tukey's test at 5% probability.

Results and Discussion

Morphological characterization of fruits and seeds

The ripe *A. colubrina* fruit is dehiscent and brown colored (Fig. 1A). The average number of seeds per fruit is 10. The average fruit length is 15 cm and the 1000-seed weight is 118 g. The seeds show length average of 1.50 cm, width or diameter average of 1.62 cm and thickness average of 0.83 mm.

The seed is morphologically flat and shiny, with a narrow wing (Fig. 1B). The embryo, which is light yellow, consists of two flat cotyledons, foliaceous, with an approximately circular contour and a basal cleft that ends at their insertion point in the hypocotyl-radicle axis (Fig. 1C), which is short and straight, exposed by the basal incision of cotyledons.

The results obtained in this study are in agreement with those reported for the same species by Espinoza (2004), with fruit length between 18 cm and 20 cm in lots collected in successive years, while the average width was 2.1 cm in samples from Cochabamba, Bolivia, at altitudes between 1,990 m and 2,100 m. The same author reported that seed diameter varied between 1.66 cm and 1.72 cm.

The observation of the embryonic axis to the scanning electron microscope showed that the plumule is differentiated, with delimited leaflets (Fig. 1D). The plumule is folded over the long and arcuate epicotyl, at an angle of about 180°, as described by Oliveira (1999). Numerous multifoliate leaflets are differentiated in the plumule, where rachis and leaflets are visible (Fig. 1D). The protoderm is glabrous in the hypocotyl-radicle axis, with numerous trichomes in differentiation on epicotyl, pluricellular glandular type (Fig. 1E). Cotyledons are thin and rich in lipids (Fig. 1F), and are not detected the presence of starch. The embryonic axis of *A. colubrina* seeds is differentiated; the cells are rich in lipids and with little amount of starch grains (Figs. 1G, 1H and 1I).

The obtained results corroborate those by Nascimento et al. (2007), who observed the presence of a non-specific material reacting with Schiff's reagent with globular characteristics in the cell cytoplasm of cotyledons from germinated *A. colubrina* seeds with 1.0 mm radicle length. According to the authors, it is probably glycoproteins and not starch, since the lugol reaction was not positive for this polysaccharide. This result is in accordance with those obtained in this study for *A. colubrina*, since the presence of starch in cotyledons was not detected.

Oliveira (1999) described the embryos of two species from the *Mimosoideae* subfamily, especially the embryonic axis of *A. macrocarpa* (Benth.) as having differentiated plumules, when there are distinguishable leaf primordia, leaflet, stipule and/or petiole differentiation may occur. The same author observed the presence of numerous differentiated non-glandular and glandular trichomes for *Inga urugiensis*, also taniferous, with idioblasts both in the protoderm and in the fundamental meristem, especially in the cortical region.

Effect of temperature and luminosity on seed germination

There was no significant effect for the triple interaction between seed lots, luminosity conditions and thermal regimes for all analyzed variables (Table 1). The significant double interaction between luminosity and temperature

Table 1. Mean values of germination percentage (G%), dead seeds (DS%) and Germination Speed Index (GSI) in lots A and B of *A. colubrina* seeds, under different lighting conditions and thermal regimes

Treatments	G (%)	DS (%)	GSI
Lots			
A	93 a	6 b	19.20 a
B	88 b	10 a	16.16 b
Luminosity			
Presence	90 a	9 a	17.45 a
Absence	91 a	7 a	17.91 a
Temperatures (°C)			
25	81 b	17 a	17.16 b
30	96 a	6 b	20.59 a
15-25	91 a	6 b	17.84 b
20-30	93 a	3 b	15.12 c

Means followed by the same lowercase letter in each column are not significantly different using Tukey's test at $p < 0.05$.

conditions was verified only for the percentages of normal and abnormal seedlings (Table 2).

With respect to germination, the constant temperature of 30 °C and alternating regimes of 15-25 °C and 20-30 °C provided a higher germination percentage in the seeds from

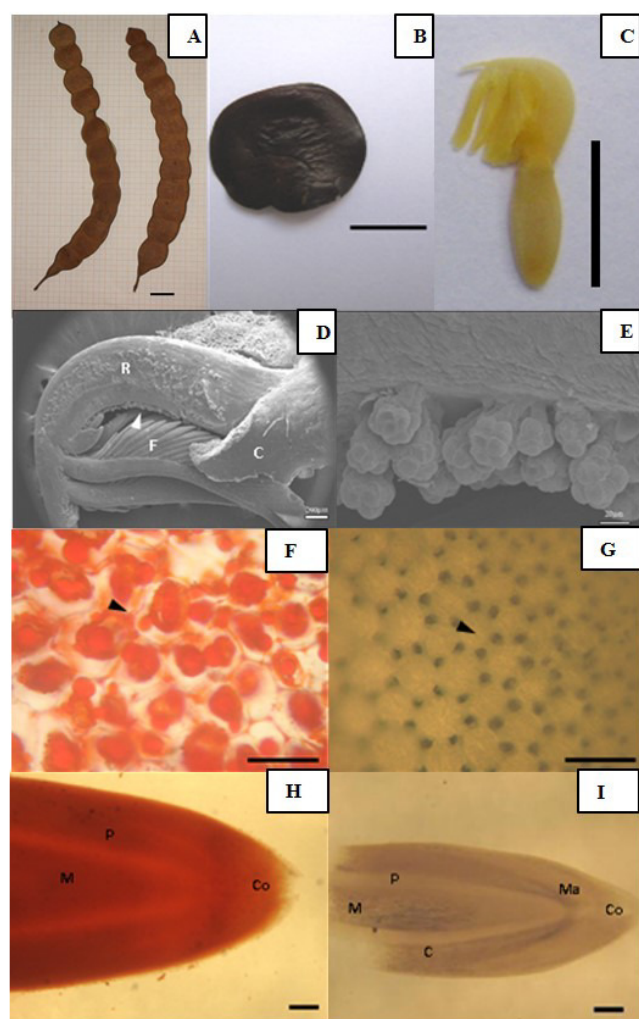


Fig. 1. Appearance of fruits (A); fruits with whole seed husk (B); and embryo axis (C); Embryonic axis scanning of *A. colubrina* seeds (D); plumule with trichomes in the lower part of the pluto-hypocotyl axis (E); (arrow: differentiating trichomes, F: leaflets; R: rachis; C: cotyledons attached to the axis). Photomicrography of cotyledonary (F and G) and longitudinal sections of the embryonic axis (H and I) of *A. colubrina* seeds stained with Sudan III (F and G) and Lugol (H and I) (arrows: lipid and starch grains, M: fundamental meristem, P: procambium, Co: root cap, C: cortex, Ma: apical meristem). Bar (A) = 2.0 cm, bar (B) = 1.0 cm, bar (C) = 0.5 cm, bars (F and G) = 0.5 μ m and bars (H and I) = 20 μ m

lot A, but there was no effect on luminosity conditions. It is observed that the temperature of 25 °C yielded a higher percentage of dead seeds from lot B (Table 1). Regarding GSI, a higher vigor was verified in seeds from lot A at 30 °C, regardless of luminosity conditions (Table 1). The highest percentage of normal seedlings was verified in seeds from lot A, and abnormal seedlings in seeds from lot B (Table 2).

Through the data obtained for double interaction between luminosity and temperature conditions, it was verified that the percentage of normal seedlings was superior at a constant temperature of 30 °C in the presence of light and alternating temperature of 20-30 °C in the absence of light, without significant differences for the other treatments. For the percentage of abnormal seedlings, the lowest value was observed at the alternating temperature of 20-30 °C; the other treatments did not differ among themselves (Table 3). Compared with every luminosity condition, when seeds were submitted to the presence or absence of light, a lower percentage of normal seedlings occurred at a constant temperature of 25 °C (Table 3). Regarding the percentage of abnormal seedlings, both in the presence and absence of light, the highest percentage was observed at 25 °C.

According to Ramos and Varela (2003), the ideal germination temperature generally varies within the temperature range found at the site and in the ideal period for the emergence and establishment of seedlings. In studies performed with *A. colubrina*, it was observed that the percentage of seedling formation did not show statistical differences between the analyzed temperatures (20, 25, and 30 °C constant); however, they concluded through the analyzed variables that the temperature of 20 °C is the more suitable for seed germination and seedling development (Paim et al., 2016). The obtained information proves that *A. colubrina* seeds can be positively or negatively influenced by light or behave indifferently to it, concluding that they do not have photosensitivity.

Effect of substrate on seed germination

On paper roll, a higher germination percentage (radicle protrusion) and normal seedlings were observed in the seeds from lot B. For the other substrates, there were no significant differences among seed lots (Table 4). Regarding GSI, a higher value was verified for the seeds from lot A using sand and paper roll substrates, and there were no differences in the amount the other substrates for both lots. A higher vigor was observed in lot A when sand was used and within lot B, also on sand and commercial substrate (Table 4). Abnormal seedlings and dead seeds were observed on paper roll (Table 5).

The characteristics of shoot length, root system length and dry matter of the root system were influenced by the double interaction between substrates and seed lots (Table 6). Seeds from lot A, germinated on paper roll and on sand, showed larger shoot length, unlike seedlings of germinated

Table 2. Percentage of normal seedlings (NS%) and abnormal seedlings (AS%) in lots A and B of *A. colubrina* seeds

Lots	NS (%)	AS (%)
A	85 a	10 b
B	69 b	17 a
CV (%)	11.95	6.38

Means followed by the same lowercase letter in each column are not significantly different using Tukey's test at $p < 0.05$.

Table 3. Percentage of normal and abnormal seedlings for interaction between different light conditions and thermal regimes in lots A and B of *A. colubrina* seeds

Temperatures (°C)	Luminosity	
	Presence	Absence
	Normal seedlings (%)	
25	62 aB	55 aC
30	85 aA	75 bB
15-25	86 aA	87 aA
20-30	77 bA	88 aA
	Abnormal seedlings (%)	
25	20 aA	28 aA
30	7 aBC	8 aB
15-25	10 aC	5 aB
20-30	18 aAB	9 bB

Means followed by the same lowercase letter in each line and the same uppercase letter in each column are not significantly different using Tukey's test at $p < 0.05$.

Table 4. Percentage of germination (G%), germination speed index (GSI) and normal seedlings (NS%), for different substrates in lots A and B of *A. colubrina* seeds

Substrates	Lots	
	A	B
	Germination (%)	
Paper roll	73 bB	94 aA
Paper sheet	95 aA	100 aA
Commercial substrate	96 aA	94 aA
Sand	97 aA	98 aA
CV (%) = 5.88		
	GSI	
Paper roll	21.21 aAB	13.46 bB
Paper sheet	14.42 aC	14.00 aB
Commercial substrate	17.47 aBC	19.58 aA
Sand	22.79 aA	19.46 bA
CV (%) = 11.51		
	Normal seedlings (%)	
Paper roll	67 bB	88 aA
Paper sheet	83 aA	88 aA
Commercial substrate	94 aA	91 aA
Sand	93 aA	96 aA
CV (%) = 8.35		

Means followed by the same lowercase letter in each line and the same uppercase letter in each column are not significantly different using Tukey's test at $p < 0.05$.

Table 5. Percentage of abnormal seedlings (AS%) and dead seeds (DS%) for different substrates in lots A and B of *A. colubrina* seeds

Substrates	AS (%)	DS (%)
Paper roll	7 a	15 a
Paper sheet	0 b	2 b
Commercial substrate	3 ab	4 b
Sand	2 ab	2 b

Means followed by the same lowercase letter in each column are not significantly different using Tukey's test at $p < 0.05$.

seeds from other substrates. Evaluating the same variable within lot B, a better behavior was observed for seedlings in commercial substrate, probably due to the physical constitution of this substrate, which allows better oxygenation conditions to the seedlings. When the seed lots were compared within each substrate, a larger paper roll length was verified for lot A and in commercial substrate for lot B, not differing for the other substrates (Table 6).

Regarding root system growth, length in lot A was greater on paper roll and commercial substrate and, within lot B, a higher value was observed in commercial substrate. In the comparison of seed lots within each substrate, it was verified that seeds from lot A showed a longer root length than seeds from lot B in paper roll, and there were no significant differences for the other treatments (Table 6). Regarding dry matter of the root system, there was no

Table 6. Root length (RL), shoot length (SL) and root dry matter (mg) (RDM) for different substrates in lots A and B of *A. colubrina* seeds

Substrates	RL		SL		RDM	
	Lots					
	A	B	A	B	A	B
Paper roll	13.18 aA	1.27 abB	12.33 aA	7.63 bB	18.2 aA	7.7 bB
Paper sheet	9.92 bA	9.36 bA	7.20 bA	6.35 bA	15.6 aA	15.8 aA
Commercial substrate	11.14 bB	12.69 aA	10.73 aA	11.28 aA	12.9 aB	17.4 aA
Sand	9.56 bA	10.71 bA	6.48 bA	6.15 bA	14.8 aA	16.1 aA

Means followed by the same lowercase letter in each line and the same uppercase letter in each column are not significantly different using Tukey's test at $p < 0.05$.

Table 7. Shoot dry matter (SDM), root dry matter (RDM) and ratio (SDM/RDM) of normal seedlings for different substrates in lots A and B of *A. colubrina* seeds

Substrates	SDM	SDM/RDM
Paper roll	74.7 bc	0.1842 b
Paper sheet	72.2 c	0.2852 a
Commercial substrate	104.9 a	0.1817 b
Sand	101.3 ab	0.1758 b
CV (%)	91.70	92.52

Means followed by the same lowercase letter in each column are not significantly different using Tukey's test at $p < 0.05$.

significant difference among different substrates for lot A and, for lot B, the lowest value was observed in paper roll; the other treatments did not differ among themselves. Comparing the seed lots within each substrate, there was a higher dry matter of the root system for seeds from lot A on paper roll and for seeds from lot B in commercial substrate, with no differences among seed lots within the substrates on paper and sand (Table 6).

Regarding shoot dry matter and its relation with the root system, the double interaction between seed lots and different substrates was not significant. A higher shoot dry matter accumulation was obtained in seedlings germinated in commercial substrate, while lower values were observed in substrate on paper. The relationship between root and shoot dry matter was higher in substrate on paper than in the other substrates (Table 7). This test can be used to evaluate seedling growth and to determine, with greater precision, the transfer of dry matter from the reserve tissues to the embryonic axis in the germination phase, originating seedlings with higher weight due to the higher dry matter accumulation (Nakagawa, 1999).

Kissmann *et al.* (2008) conducted studies with the objective of evaluating temperature and ideal substrates for the germination of *Adenantha pavonina* L. seeds. These authors observed higher germination values in substrates on paper and paper roll averaging 86%, and no significant differences were observed in the dry weight of seedlings, averaging 1.08 g, submitted to different temperatures and substrates. According to Figliolia *et al.* (1993), the use of vermiculite proved to be inadequate for *M. caesalpiniifolia* seeds. The authors also describe that seeds from this species are indifferent to light during germination, and the latter result is similar to that obtained in this study for *A. colubrina* seeds.

Amaro *et al.* (2006) studied the influence of light and temperature on the germination of janaguba (*Himatanthus drasticus* (Mart.) Plumel.) seeds and verified that these seeds show neutral photoblastism; temperatures of 20 °C and 25 °C, combined with constant dark and light/dark, and

temperatures of 30 °C and 20-35 °C, combined with light/dark, are the most favorable conditions for germination. The combination of temperatures of 25 °C and 30 °C and the absence of light increase the speed and reduce the average germination time of this species.

Melo *et al.* (2005) evaluated the effect of different substrates on the germination of *Anadenanthera colubrina* (Vell.) Brenan seeds under laboratory conditions and observed that the highest seed germination percentage occurred in the substrates between sand and vermiculite at 30 °C and 12-hour photoperiod. They also found that the substrate between paper sheet showed the worst performance, with the lowest numbers of germinated seeds and the highest number of abnormal seedlings. These authors reported that, although all the substrates used have the partial restriction of light incidence on the seeds as a common characteristic, the difference can be explained by the low thermal conductivity of paper sheet, which caused the germination percentage of this treatment to be significantly lower, when compared to the others.

Conclusions

The seminal anatomy of *A. colubrina* is typical of the *Mimosoideae* subfamily. The number of seeds per fruit is 10 on average and the 1000-seed weight ranges 118 g. *A. colubrina* seeds are indifferent to light and the highest vigor was verified at 30 °C. Sand and commercial substrates are efficient in initial seedling development.

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