Full Length Research Paper

Seed quality evaluation by tetrazolium staining during a desiccation study of the recalcitrant seeds of *Carapa guianensis* Aubl. and *Carapa surinamensis* Miq. - Meliaceae

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Crabwood, a popular name of several pantropical timber species, has become increasingly important for its seed oil of pharmaceutical and cosmetic use. Due to the recalcitrant character of the seeds, plantations are limited. The aim of this study was to develop a tetrazolium (TZ) staining protocol and validate viability staining with germination tests. Seed preparation was standardized in order to localize and cut the tiny embryonic axis longitudinally, which is inserted in the fused cotyledonal seed mass. Staining intensity was determined by testing different concentrations of TZ solution (0.05, 0.10, 0.25 and 0.50%) at three temperatures (25, 30 and 35°C) during a period of up to 6 h. Taking into account the large seed size, costs and working time, a solution of 0.10% TZ at 30°C for 3 h was considered appropriate for both species. The method was validated with seeds of different qualities (between 0 and 90% germination capacity), obtained by controlled drying over a fan. The desiccation revealed initial damage near the seed surface close to the radicle meristem. Images of the stained seeds were classified in four viability classes and were re-evaluated with the germination results (radicle ≥ 0.5 cm and normal seedlings). The proposed method for tetrazolium staining was effective in assessing seed viability of both species.

Key words: Andiroba, *Carapa procera* D.C., water content, embryonic axis, germination, crabwood.

INTRODUCTION

Amazonia is known for a high diversity of species with commercial importance, some widely exploited due to their multiple uses are submitted to deforestation and non-sustainable exploitation (Souza et al., 2008). Among these are the species of the genus *Carapa* (Meliaceae), known popularly as crabwood, andiroba, roba-mahogany,

among others. These species occur in the Neotropics, Africa and India (Kenfack, 2011), with similar use and extraction of the seed oil on all continents (Weber et al., 2010). A recent taxonomic revision restricted the occurrence of *Carapa procera* D.C. to the African continent, and according to the same study, three species, *Carapa guianensis* Aubl., *Carapa surinamensis* Miq. and *Carapa vasquezii* Kenfack (Kenfack, 2011) exist in the Brazilian Amazon region. In this way, the results published up to 2011 on *C. procera* seeds collected in the Amazon were attributed to *C. surinamensis.*

Andiroba seeds are large and individual seed weight averages 25 g for *C. guianensis* and 16 g for *C. surinamensis* (Ferraz et al., 2002). The seeds are recalcitrant (Connor et al., 1998) and this characteristic is one of the causes of poor seed trade. Recalcitrant seeds are generally considered to germinate immediately after dispersal (Daws et al., 2005). Under nursery conditions, *C. guianensis* seeds needed between 26 and 180 days for emergence, and *C. surinamensis* between 11 and 38 days, both showed fast subsequent development, and in about ten days the shoot length until the first expanded leaves reached 40 and 25 cm, respectively (Ferraz et al., 2002). Seed testing rules (Brasil, 2009; ISTA, 2015) evaluate germination until the development of a normal seedling. Thus, seed testing in the laboratory is difficult.

The internal seed morphology reveals a uniform seed reserve tissue, formed by the conferruminate, that is, fused cotyledons. A visual distinction between the two cotyledons is not possible (Harshberger, 1902). The small embryonic axis (length ca. 2 mm) is inserted in the cotyledonal tissue near the micropyle on the tetrahedron face of the seed (Ferraz et al., 2002). Some seeds of *C. surinamensis* may have several embryonic axes (Ferreira et al., 2017).

Seed quality assessment of recalcitrant seeds, or those with slow germination (>60 days), can be done with viability tests (Brasil, 2009). The best known is topographic staining with tetrazolium (2,3,5-triphenyl tetrazolium chloride - TZ) (Lakon, 1949). The respiratory activity of seeds in living tissue, specifically hydrogen ions (H^+) released by dehydrogenases, reduce soluble and uncoloured tetrazolium to a red insoluble compound, known as triphenylformazan. Thus metabolically active tissues present red staining while dead or damaged tissues remain unstained.

Viability tests generally overestimate germination results and should be validated with germination tests. Species-specific protocols may be necessary. The embryonic axis has to be evaluated and, depending on seed morphology, the vital parts have to be visible. Seeds may need conditioning in water, and the concentration of the TZ solution, immersion time and temperature has to be known, as well as tissue consistency and the location and size of possible lesions in relation to seed morphology (França-Neto et al., 1998; Brasil, 2009; Fogaça et al., 2011).

Instructions for TZ concentrations for 154 species, including agricultural and forestry species, were published in the Brazilian Seed Testing Rules, and solutions of 0.5 and 1.0% were recommended in most cases (Brasil, 2009). The International Seed Testing Association (ISTA) recommends solutions of 1% TZ and 30°C as immersion temperature during 18 to 48 h; for the 120 forestry species studied, emphasis is given to seed preparation, to permit the evaluation of the embryonic axis (Leist and Krämer, 2011). *Carapa* spp. have several seed characteristics which would need fast quality assessment, such as large recalcitrant seeds, slow and not uniform germination, and tall seedlings, however no information on TZ staining was found.

In this sense, the aim of this study was (a) to elaborate a protocol for seed preparation and staining of *C. guianensis and C. surinamensis* with tetrazolium solution, and (b) to validate the TZ staining with germination tests.

MATERIALS AND METHODS

Collection area

The seeds of *C. guianensis* and *C. surinamensis* were collected in March 2014 during the natural seed dispersal in a 40-year-old plantation at the Experimental Forestry Station of the Brazilian National Institute for Amazonian Research (Instituto Nacional de Pesquisas da Amazônia - INPA), located at km 45 on the BR-174 highway north of Manaus (02° 35' 55.5" S and 60° 02' 14.8" W), Amazonas State. Due to the desiccation intolerance of the seeds, fruits and seeds were transported immediately after collection in semi-permeable plastic bags to the seed laboratory of INPA.

Processing

The capsule-type fruits were opened manually by removing the fruit valves. Seeds with visible damage were eliminated. After submersion in water for 24 h to drown potential larvae of *Hypsipyla* sp., a lepidopteran known as a seed borer, the seeds were superficially dried above wires at room temperature for about 2 h.

Adequacy of the tetrazolium test

Preliminary tests aimed to determine the tetrazolium concentration, temperature and immersion time. After longitudinal sections, the seeds were preconditioned in distilled water for 24 h at 25°C (Brasil, 2009). Due to the size and morphology of the seed, it was possible to cut them a second time, in order to observe the staining at four TZ concentrations (0.05, 0.10, 0.25 and 0.50%) on the same seed. Each section was totally immersed in the TZ solution in a 50 mL plastic cup, covered with plastic film to reduce evaporation, and maintained in the dark. Three immersion temperatures were compared: 25, 30 and 35°C (± 2°C), using germination chambers (Fanem®). In each of the twelve conditions (four concentrations × three temperatures) 24 seeds were observed, totalling 288 samples. The staining intensity of the seeds was assessed hourly. The colours were classified in intensity levels according to the plant tissue colour chart (Munsell Color Charts, 1977) without staining (natural colour of tissue 2.5Y ± 8/2), very weak (2.5R 8/4), weak (2.5R 7/4), adequate (2.5R 7/6 and 7/8) and excessive staining (2.5R 5/8 and 5/10).

Seed preparation and exposure of embryonic axis

Seeds were transversely sectioned into two parts (Figure 1A and B). The distal part was used for moisture content determination, while the part with the embryonic axis was destined for tetrazolium staining. The woody seed coat has to be removed carefully with a metal spatula. A circular light brown area is differentiated from the brown seed surface. The centre of the circle has a small elevation and papyraceous tissues, slightly darker brown than the circle area, covering the axis. These tissues must be carefully removed to reveal the basal part of the embryonic axis (Figure 1C). A longitudinal cut through the axis permits its exposure to the TZ solution (Figure 1D).

Viability validation

Seeds of different germination capacities were obtained by controlled drying during 0, 1, 3 and 7 days at room temperature (25 ± 2°C, ± 60% RH), at 70 cm above an air circulator (Arno WWB3/5). The seeds were maintained in nylon mesh nets for fruits, which allowed circulation of air between the seeds. Each net contained 120 seeds, with four repetitions of 15 seeds for viability staining and the same amount for germination tests. For TZ staining, seeds were prepared as described previously and, based on results of the preliminary tests, immersed in 0.10% TZ for 3 h at 30°C and rinsed afterwards with distilled water.

TZ staining was observed under a stereo microscope (Leica S8 APO). Images of each seed were recorded (Leica DFC295), however they were assessed only when the germination results were available. Staining evaluation was based on França-Neto (1999), where bright red or pink colours were living tissue, and milky white or yellowish colours, dead tissue. These colours were identified with the plant tissue colour chart (Munsell Color Charts, 1977) as 2.5R 7/6 and 7/8 (living tissue), 2.5Y 7/6, 7/8 and 7/10 (dead tissue). Seeds with no red or pink colouration were easily recognized as dead, nevertheless the ones which still showed some reddish colours were difficult to evaluate. Thus, the images of all seeds were organized according to their colouration patterns, considering primarily the embryonic axis and secondly the tissue of the fused cotyledons. Four classes with decreasing red colouration could be established. Afterwards, the results of the germination tests were matched with the TZ evaluation, considering class 1, 2 or 3 as viable seeds. The relation between germinability and viability was evaluated with a Pearson correlation coefficient (r).

Seed moisture content

Determined gravimetrically after drying at 105°C (Brasil, 2009); the samples were weighted every 24 h until mass stabilization (0.001 g) and the moisture content was expressed as percentage of fresh weight. Moisture content was assessed for each seed of the viability test, using the distal portion (about 10 g), sectioned twice for faster drying. The results were evaluated in four repetitions of 15 seeds, for each treatment.

Germination test

The seeds were sown in plastic trays (30 × 22 × 7 cm) in moistened vermiculite of medium granulation (Brasil Minérios®) (2 g water / 1 g vermiculite). The trays were covered with transparent polyethylene bags (40 × 60 cm and 8 mm) to reduce evaporation. The test was performed at 25°C (± 2°C) with 12 h photoperiod (cool white fluorescent light, PAR 015 × 10 µmol·m^{-2}·s^{-1}).

The first germination criterion, protrusion of the radicle (≥ 0.5 cm), was assessed daily. Germinated seeds were transferred to the
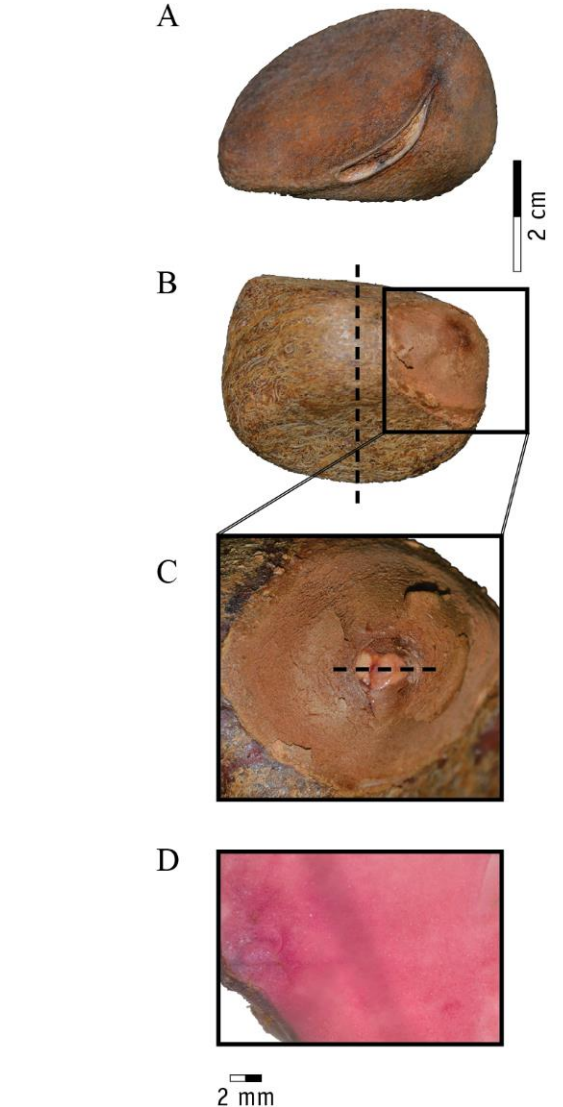

Figure 1. Seed preparation of crabwood seeds, exemplified with *C. surinamensis*, to expose the embryonic axis to the tetrazolium solution. (A) Seed with seed coat and hilum view. (B) Seed without seed coat and same view as above, the dotted line indicates the first cross section (C). After removal of the papyraceous tissues, the basal end of embryonic axis is visible in the centre of the light brown circle; the horizontal dotted line indicates the second section. (D) The embryonic axis with about 2 mm length after TZ staining.

nursery in larger trays (57 × 25 × 17 cm) and development monitored until the expansion of the first pair of leaves (second germination criterion). A normal seedling, according to seed testing standards, had radicle and shoot with perfect development and showed high probability of establishment under favourable conditions (Brasil, 2009). The results were expressed as percentage of germinated seeds for each criterion.

Table 1. Immersion time at three temperatures and four concentrations of tetrazolium solution (TZ) to achieve four qualities of tetrazolium staining in *Carapa* sp. seeds; no-uniform staining is marked with (*).

Temperature (°C)	TZ (%)	Immersion time (hour)					
		1	2	3	4	5	6
25	0.05	None	Very weak	Very weak	Very weak	Weak	Adequate
	0.10	None	Very weak	Very weak	Weak	Adequate	Adequate
	0.25	Very weak	Very weak	Weak	Adequate	Adequate	Excessive
	0.50	Very weak	Weak	Adequate	Excessive	Excessive	Excessive
30	0.05	None	Very weak	Weak	Weak	Adequate	Adequate
	0.10	Very weak	Weak	Adequate	Adequate	Excessive	Excessive
	0.25	Very low	Weak	Adequate	Adequate	Excessive	Excessive
	0.50	Very low	Weak	Adequate	Excessive	Excessive	Excessive
35	0.05	None	Very weak	Weak	Adequate*	Adequate*	Excessive*
	0.10	Very low	Weak	Adequate*	Adequate*	Excessive*	Excessive*
	0.25	Very low	Weak	Adequate*	Adequate*	Excessive*	Excessive*
	0.50	Weak	Adequate*	Excessive*	Excessive*	Excessive*	Excessive*

None (natural tissue colour 2.5Y ± 8/2); very weak (2.5R 8/4); weak (2.5R 7/4); adequate (2.5R 7/6 and 7/8) and excessive (2.5R 5/8 and 5/10) colour indication in accordance to Munsell Color Charts (1977).

Statistical analyses

The experimental design was completely randomized. Normality was tested with the Shapiro-Wilk test for germinability and viability, and the homogeneity of variances with Levene's test at 0.01% significance. Results were compared by analysis of variance (ANOVA) followed by comparison of the means through Tukey test at 5% probability, using SISVAR (system for variance analysis) (Ferreira, 2011). Pearson correlation coefficients (r) between tetrazolium and germination tests (radicle protrusion and normal seedling) were calculated. The significance of r values was determined by the *t*-test at 1% probability (Pimentel-Gomes, 2000).

RESULTS AND DISCUSSION

The immersion time to achieve an adequate staining with TZ depended on the immersion temperature, the TZ concentration and using less TZ being more economical. Adequate staining was achieved at 25°C after 3 h with 0.50% TZ, while less-concentrated solutions needed 4 h (0.25%), 5 h (0.10%), and 6 h (0.05%). At 30°C, adequate staining was achieved after 3 h in concentrations of 0.10, 0.25 and 0.50%, the staining remained adequate with 0.10 and 0.25% up to the fourth hour before becoming excessive (Table 1). At 35°C, staining at all concentrations was not uniform and not appropriate for viability evaluation, as staining uniformity and tone are important for reliable interpretation of the results (Moore, 1985; Bhering et al., 2005). Intense staining may also result in misinterpretation, as shown for *Enterolobium contortisiliquum* (Vell.) Morong seeds (Nogueira et al., 2014).

The individual assessment of seed viability, with a solution of 0.10% TZ, needs about 20 ml of solution per seed, totalling 2 L for 100 seeds, corresponding to 2 g of TZ. The assessment after 3 h at 30°C allows running the test in a working day, taking into account the time consumed in seed preparation and staining evaluation with a magnifying glass.

The instructions of ISTA indicate 1.0% TZ at 30°C for forestry species with variation only in the immersion time, which was predominantly 18 h for 90% of the 121 species studied (Leist and Krämer, 2011). The instructions for Seed Testing in Brazil (Brasil, 2009) recommend concentrations between 0.5 and 1.0% TZ, immersion periods from 6 to 24 h at 30°C in average, and according to the species, recommended temperature ranges between 20 and 40°C.

The importance of considering the amount of TZ used and the duration of the test was reported in other studies (França-Neto et al., 1998; Dias and Alves, 2008; Corte et al., 2010), which revealed the possibility of using lower concentration without reducing the quality of the results. A concentration of 0.07% TZ was recommended, e.g., for *Enterolobium contortisiliquum* (Vell.) Morong (Nogueira et al., 2014), *Poecilanthe parviflora* B. (Pinto et al., 2008) and *Mouriri elliptica* Mart. (De Lima et al., 2016). For other species, 0.50% TZ was reported, e.g., for *Ceiba speciosa* (A.St.-Hil.) Ravenna (Lazarotto et al., 2011) and *Acrocomia aculeata* (Jacq.) Lodd. ex. Mart. (Ribeiro et al., 2010) and 0.075% for *Glycine max* (L.) Merril (Zuffo et al., 2015). A concentration equal to this study (0.10% TZ) was used for seeds of *Schizolobium parahyba* Vell. Blake (Fogaça et al., 2011) and *Peltophorum dubium* (Sprengel) Taubert (Oliveira et al., 2005).

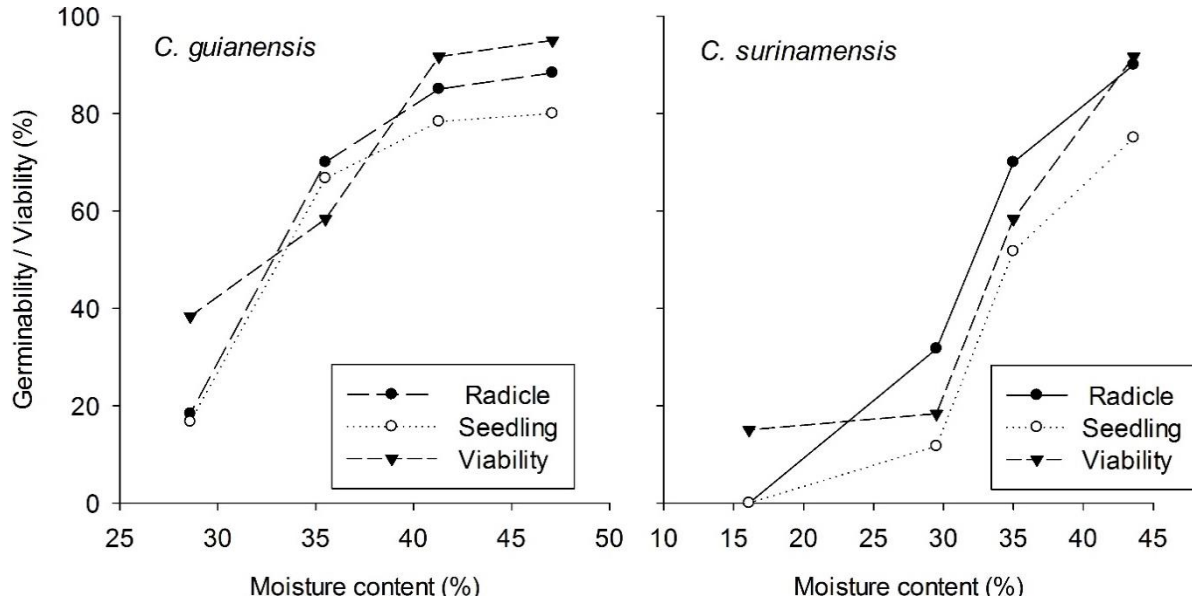The immersion time varied by tree species. Some required a very short time, such as 90 min for

Figure 2. Germinability (radicle protrusion and normal seedling) and Viability (TZ staining) in relation to seed moisture of the recalcitrant seeds of *C. guianensis* and *C. surinamensis*.

Poecilanthe parviflora Benth. (Pinto et al., 2008), others a similar time to this study, from 3 to 4 h of immersion, e.g., *Enterolobium contortisiliquum* (Vell.) Morong (Nogueira et al., 2014), *Schizolobium parahyba* Vell. Blake, *Copaifera langsdorffii* Desf. (Fogaça et al., 2011), *Acrocomia aculeata* (Jacq.) Lodd. ex. Mart. (Ribeiro et al., 2010) and *Glycine max* (L.) Merril (Zuffo et al., 2015). For *Ricinus communis* L. a staining period of 6 h is recommended (Oliveira et al., 2014a). However, most require a longer period with the results assessed the next working day (Leist and Krämer, 2011; Abbade and Takaki, 2014).

Different seed qualities of both *Carapa* species were obtained by a controlled drying of the recalcitrant seeds. A similar approach to obtain different seed qualities were done recently for *Acrocomea aculeata* (Jacq.) Lood. ex Mart. (Rubio Neto et. al., 2015). Initial moisture content was 47.1% (*C. guianensis*) and 43.6% (*C. surinamensis*) and both species showed high percentages of radicle protrusion and normal seedlings (*C. guianensis* 88.3 and 80.0% and *C. surinamensis* 90.0 and 75.0%, respectively). Drying for seven days reduced the moisture content to 28.6% (*C. guianensis*) and 16.1% (*C. surinamensis*) and also the percentage of radicle protrusion (to 18.3%) and normal seedlings (to 16.7%) in *C. guianensis*, and caused the death of *C. surinamensis* seeds (Figure 2).

With the germination results, the viability classes for TZ staining were validated and consolidated for both species (Figures 3 and 4). The staining pattern was similar between the species; however, the staining of *C. guianensis* seed reserves was more homogenous than of *C. surinamensis*. Evaluating strictly the embryonic axis, a uniform colouring in red (2.5R 7/6 and 7/8 in accordance

with Munsell Color Charts, 1977) can be considered as indicating viability for both species, in congruence with this, classes 1, 2 and 3 were defined as viable seeds, while class 4 was considered unviable (Figures 3 and 4).

Topographic staining revealed that the first area to show reduced staining caused by seed desiccation was close to the seed surface at the basal end of the radicle meristem (Viability class 3 in Figures 3 and 4). However, the seeds of class 3 showed radicle protrusion, of 70.0% (*C. guianensis*) and 31.7% (*C. surinamensis*) and developed normal seedlings at 66.7% (*C. guianensis*) and 11.7% (*C. surinamensis*) (Table 2), thus class 3 seeds were still viable, maybe with reduced vigour. Other comparative studies between TZ and germination mentioned the possibility of assessing efficiently viability and seed vigour (Bhering et al., 2005; França Neto et al., 1998; Nakagawa, 1994).

The results obtained by TZ staining showed no significant difference to the germination test for both species; normal seedling development however was more sensitive to drying than radicle protrusion as shown after 3 and 5 days of drying (Table 2). Data analysis, based on the coefficient of the linear correlation between TZ staining and both germination criteria was positive and significant (Table 3). For *C. guianensis*, the correlation between tetrazolium and radicle protrusion was 0.848, and normal seedling development, 0.792. Similar strong correlations were obtained for *C. surinamensis* between TZ staining and radicle protrusion (0.910) and normal seedling development (0.918).

A positive and significant correlation between TZ staining and field emergence or germination was earlier reported for the seeds of soybean (Barros and Marcos

Class 1: Viable seeds with uniform red brilhant colour (2.5R 7/6 and 7/8) of the embryonic axis and the seed reserves, tissues of normal aspect and firm.

Class 2: Viable seeds, red colouration similar to class 1 (2.5R 7/6 and 7/8) however with reduced colour in the centre of the embryonic axis and some spots without colour in the cotyledon mass.

Class 3: Viable seeds, red colouration similar to class 1 (2.5R 7/6 and 7/8) in ca. 50% of the cotyledonal seed reserves and reduced staining in some parts of the embryonic axis; central part of the embryonic axis whitish and radical meristem intense red or yellowish, indication tissue deterioration.

Class 4: Unviable seeds: embryonic axis and cotyledons yellowish (2.5Y 7/6, 7/8 and 7/10) with some red spots; seed reserve tissues with flaccid consistency.
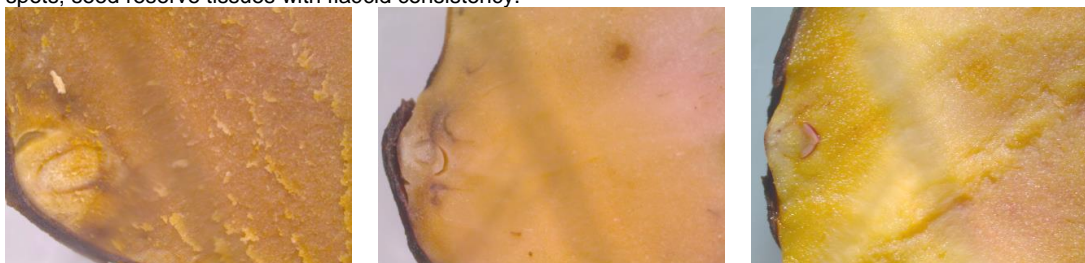
Figure 3. *C. guianensis* seeds organized according to the staining patterns in four vigour classes with three examples for each class.

Filho, 1997), zucchini (Barros et al., 2005), watermelon (Bhering et al., 2005), leucena (Costa and Santos, 2010), silk floss tree (Lazarotto et al., 2011) and palm heart (Oliveira et al., 2014b).

Conclusions

The tetrazolium test can be used to assess seed quality in the recalcitrant seeds of crabwood. The seed axis has to be exposed to the solution with only a part of the seed reserves, the majority of the seed mass can be discarded or used for other measurements, e.g., moisture content. Considering the large seed size, and the time for seed preparation and later staining evaluation, a 0.10% TZ solution with an immersion of 3 h at 30°C was appropriate for both species and the results could be correlated with radicle protrusion and normal seedling development. The desiccation study revealed initial damage near the seed surface close to the radicle meristem. The recommendations for TZ staining of crabwood seeds differed from other tree species in the

Class 1: Viable seeds with uniform red brilhant colour (2.5R 7/6 and 7/8) of the embryonic axis and the seed reserves, tissues of normal aspect and firm.

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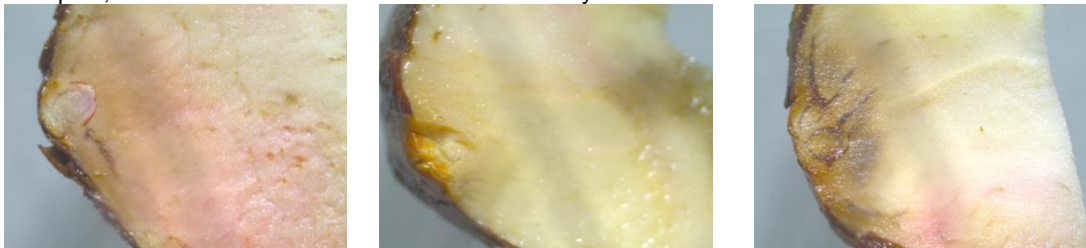

Figure 4. *C. surinamensis* seeds organized according to the staining patterns in four vigour classes with three examples for each class.

seed preparation, immersion time and temperature, and TZ concentration.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Table 2. Comparison of two germination criteria (radicle protrusion and seedling development) (%) and viability (%) assessed with tetrazolium staining of the recalcitrant seeds of *C. guianensis* and *C.surinamensis* during a controlled drying process of up to 7 days.

Species	Criterion	0 day	3 days	5 days	7 days
C. guianensis	Radicle	88.3[a]	85.0[a]	70.0[a]	18.3[a]
	Seedling	80.0[a]	78.3[ab]	66.7[a]	16.7[a]
	Viability	95.0[a]	91.7[a]	58.3[a]	38.3[a]
	CV	13.28	6.24	27.72	57.86
	LSD	23.02	10.97	35.60	27.94
	W	**0.755**	**0.010**	**0.235**	**0.342**
	F	**0.195**	**0.218**	**0.215**	**0.188**
C. surinamensis	Radicle	90.0[a]	70.0[a]	31.7[a]	0.0[a]
	Seedling	75.0[a]	51.6[a]	11.7[b]	0.0[a]
	Viability	91.7[a]	58.3[a]	18.3[ab]	15.0[a]
	CV	6.62	21.76	37.84	96.87
	LSD	11.19	25.79	15.36	9.67
	W	**0.301**	**0.856**	**0.374**	0.000
	F	**0.194**	**0.272**	**0.380**	0.028

Means followed by different letters in the columns differ by Tukey test at 0.05 of significance; W = Shapiro-Wilk test; F = Levene test. Values in bold indicate residues with normal distribution and homogeneous variances to 0.01 significance level. LSD = least significant difference. CV = coefficient of variation

Table 3. Pearson correlation coefficients between the results of radicle protrusion (R), normal seedling development (NS) and the tetrazolium staining (TZ) for *C. guianensis* seeds and *C. surinamensis.*

Species	Test	TZ	R	NS
C. guianensis	TZ	1	0.848**	0.792**
	R		1	0.961**
	NS			1
C. surinamensis	TZ	1	0.910**	0.918**
	R		1	0.990**
	NS			1

**Correlation is significant at the 0.01 level (2-tailed).

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