

Viability of recently harvested and stored *Xylopia aromatica* (Lam.) Mart. (Annonaceae) seeds¹

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ABSTRACT - *Xylopia aromatica* is a native species from Brazil's "Cerrado", recommended for restoration ecology and also as a medicine. Its seeds have embryos with morphophysiological dormancy, making nursery propagation difficult. The objective of this study was to verify the efficiency of X-ray and tetrazolium tests for evaluating the viability of three seed lots, stored for different periods. All seeds were X-rayed (13 kV, 350 seconds) and samples used for tetrazolium and germination tests. In the tetrazolium test, seeds were submitted to six treatments at two temperatures (25 and 30 °C) with imbibition in distilled water and immersion in three concentrations of tetrazolium solution (0.5, 0.75 and 1%) at the two imbibition temperatures. Seeds for the germination test were placed for imbibition in distilled water and a 500 ppm Promalin® (6-Benzyladenine + GA₄ + GA₇) solution and later sown in sterilized sand. The embryo could not be observed with the X-ray test. However, those seeds observed with an undamaged endosperm did not differ in the percentages of seeds with firm and stained endosperms observed in the tetrazolium test for all the lots. The tetrazolium test is efficient for evaluating seed viability, principally if imbibed at 30 °C and immersed in a 0.5% solution at 30 °C.

Index terms: "pimenta-de-macaco", forestry seed, image analysis, seed quality.

Viabilidade de sementes de *Xylopia aromatica* (Lam.) Mart. (Annonaceae) recém colhidas e armazenadas

RESUMO - *Xylopia aromatica* é uma espécie nativa do Cerrado, indicada para recuperação de áreas degradadas e uso medicinal. Suas sementes possuem embriões com dormência morfofisiológica, dificultando a propagação em viveiros. Este estudo teve como objetivos verificar a eficiência dos testes de raios X e de tetrazólio na avaliação da viabilidade de três lotes de sementes, com diferentes períodos de armazenamento. Todas as sementes foram radiografadas (13 kV, 350 segundos) e, posteriormente, amostras utilizadas nos testes de tetrazólio e de germinação. As sementes foram submetidas a seis tratamentos, duas temperaturas (25 e 30 °C) de embebição em água destilada e imersão em três concentrações da solução de tetrazólio (0,5, 0,75 e 1%) nas mesmas temperaturas. Para germinação, as sementes foram colocadas para embeber em água destilada ou em solução de Promalin® (6-Benzyladenine + GA₄ + GA₇) a 500 ppm e semeadas em areia esterilizada. Os embriões não foram visualizados pelas imagens radiográficas. Sementes sem danos no endosperma não diferiram das porcentagens de sementes com endospermas firmes e coloridos observados no teste de tetrazólio para todos os lotes. O teste de tetrazólio é eficiente para avaliar a viabilidade das sementes, principalmente quando são embebidas a 30 °C e imersas em solução de 0,5% a 30 °C.

Termos para indexação: pimenta-de-macaco, análise de imagens, qualidade de semente, semente florestal.

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Introduction

Xylopia aromatica (Lam.) Mart. is a characteristic species of the Brazilian savannah (Cerrado) region with a wide distribution but only present at low population levels (Lorenzi, 1998). The seeds are around 7 mm long (Castellani et al., 2001) and embryos show morphophysiological dormancy (Sautu et al., 2007), making nursery production difficult. This species is also recommended for the recovery of degraded “Cerrado” areas in São Paulo state through the SMA Resolution n° 47/2003 (São Paulo, 2003).

The interest in propagating native forest species has grown due to the focus on environmental problems and the need to recover degraded land (Araújo Neto et al., 2003) and the demand for the seeds and seedlings of native species has encouraged research (Santos and Aguiar, 2000).

The development of quick tests to determine seed physiological quality has been one of the principal objectives of seed technologists (Cervi et al., 2009). Among those tests is the tetrazolium test, which besides evaluating viability and vigor (França-Neto, 1999; Gaspar-Oliveira et al., 2009), in some cases, permits the identification of factors which influence seed quality, including mechanical damage and that caused by drying, insects and moisture deterioration (França-Neto, 1999). This test can also be used to determine the viability of seeds which germinate slowly or which do not germinate (Piña-Rodrigues and Santos, 1988).

The X-ray test is another quick test, which evaluates seed quality and which has been used for forest species since the 1950s (Simak and Gustafsson, 1953) and 1970s (Kamra, 1976). The use of this technique in Brazil for seeds of forest species (Mondo et al., 2008; Pupim et al., 2008; Socolowski and Cicero, 2008) has been increasing.

Due to the difficulty of evaluating the physiological potential and dormancy type of *Xylopia aromatica* seeds, the objective of this study was to verify the efficiency of X-ray and tetrazolium tests for evaluating the viability of three seed lots stored during different time periods.

Material and Methods

The study was done at the Image Analysis and Seed Analysis Laboratories of the Crop Science Department of the Escola Superior de Agricultura “Luiz de Queiroz” – ESALQ/USP, in Piracicaba, São Paulo State.

X. aromatica seeds from three lots collected at different sites and at different times were used. After each collection, the seeds were separated by density in water and then

washed until the arils and sarcotesta had been removed. Seeds from lot 1 were collected in March 2009 at Mogi Guaçu (22° 22' 20" S 46° 56' 32" W), São Paulo State, and were stored for 10 months in a controlled environment (20 °C and 45% relative air humidity), inside a glass container until the beginning of the experiments. The seeds from lot 2 were collected in April 2009 at São Pedro (22° 32' 55" S 47° 54' 50" W) and Itirapina (22° 15' 10" S 47° 49' 22" W), São Paulo State, and were stored for eight months in a controlled environment (20 °C and 90% relative air humidity), inside a glass container until the beginning of the experiments. Seeds from lot 3 were collected in November 2009 from the same area as lot 1, and used in the experiments soon after collection.

The seed moisture content of each lot was determined by the oven method at 105 ± 3 °C for 24 hours (Brasil, 2009), using four replications of 25 seeds. The results were expressed as a mean percentage and were calculated based on the wet weight.

Twenty-four replications of 25 seeds were then taken from each lot ($n = 600$ seeds lot⁻¹) and all were numbered and placed individually in ice cube cells.

The seeds were distributed among cells in an acrylic plate and X-rayed with a Faxitron X Ray MX – 20 machine using X-ray film (Kodak MIN - R EV 2000, size 18 × 24 cm) placed at a distance of 57 cm from the radiation source at an intensity of 13 kV per 350 seconds. After obtaining the images, the seeds were returned to their original positions in the ice cubes.

Based on the X-ray images, the seeds were classified as undamaged, when no internal morphological damage was detected, and damaged, when the internal space was empty or there was some kind of abnormality (Figure 1).

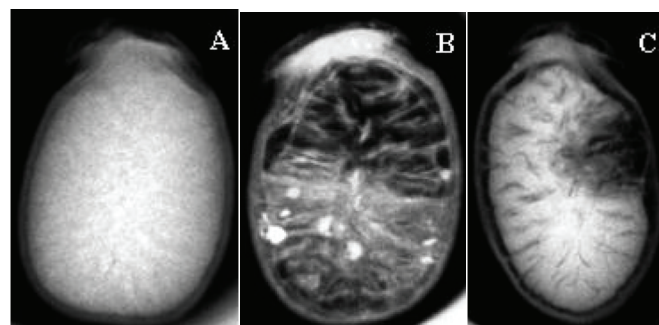


Figure 1. X-ray images of *Xylopia aromatica* seeds which are undamaged (A) and damaged, with no internal filling (B) and with abnormalities (C).

After being X-rayed, seeds were left to imbibe individually in about 3 mL of distilled water. Half of the seeds were kept at 25 °C and the other half at 30 °C, in the dark for 48 hours. The seeds were cut longitudinally

and returned to their cells, which contained approximately 3 mL of 2, 3, 5 tetrazolium triphenyl chloride solution at concentrations of 0.5, 0.75 and 1%, for 24 hours.

For the tetrazolium test evaluation, the endosperm tissue consistency (using tweezers) and staining were examined, classifying tissue as totally red stained, more than 50% red stained, less than 50% red stained and without any staining (Figure 2). The embryos were evaluated using a stereoscopic microscope and classified according to their presence, consistency and coloring (Figure 3).

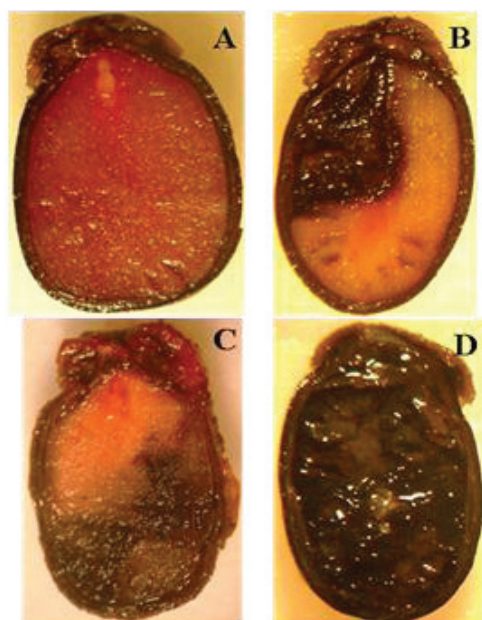


Figure 2. *Xylopia aromatica* seeds with the endosperm totally stained in red (A), more than 50% stained in red (B), less than 50% stained in red (C) and unstained (D) by the tetrazolium test.

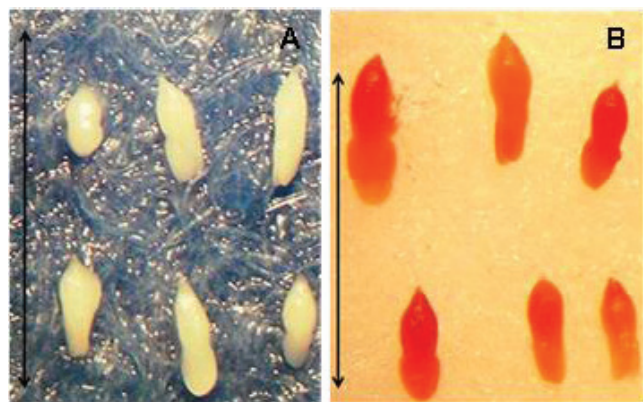


Figure 3. *Xylopia aromatica* embryos unstained (A) and stained (B) by the tetrazolium test.

Lateral bars = 2.5 mm.

Simultaneously, another eight replications of 25 seeds from each lot ($n = 200$ seeds lot⁻¹) were X-rayed and later divided into four repetitions of 25 seeds and placed to imbibe in distilled water or in a solution of Promalin® (6-Benzyladenine + GA₄ + GA₇) at a concentration of 500 mg L⁻¹, for 48 hours at 30 °C, with the objective of overcoming seed dormancy, as described by Socolowski and Cicero (2011). The seeds were then sown at 1 cm depth in 21 × 14 cm trays with 2.4 kg of sterilized sand and moistened with water up to 70% of their retention capacity; later, they were placed under cover with no temperature control to observe seedling emergence every 48 hours over 90 days. Experiments were watered daily.

The percentages and speed of emergence were calculated and also the formation of normal seedlings, according to (Labouriau and Agudo, 1987). At the end of the experiment, the percentages of abnormal seedlings and seedlings showing incomplete development were evaluated. The seedlings were considered: a) as emerged, after breaking through the substrate with 1 cm above the surface; b) as normal, when free of the seed integument; c) as abnormal, when there was rotting of the hypocotyl just below its insertion in the integument or when the cotyledons and, or apical meristem, were rotten; d) having incomplete development, when the cotyledons were still inside the integuments. Test evaluations were made every 48 hours for 90 days.

The experimental design was completely randomized. The data in percentages were transformed into arc sine $\sqrt{x}/100$. The t test at the 5% probability level was used to compare the means of each tetrazolium treatment and the X-ray images. The parameters evaluated in the germination tests, as well as the treatments means from tetrazolium test and the data for seedling emergence and normal seedling formation within each lot, were compared using one-way Analysis of Variance (ANOVA), followed by a test of the Least Standard Difference (LSD) at the 5% probability level (Vieira, 2006; Zar, 1999).

Results and Discussion

The seed moisture contents after harvesting from lots 1, 2 and 3 were 21.0, 19.9 and 14.7%, respectively. After storage, the seeds from lots 1 and 2 had water contents of 19.3 and 22.3%, respectively.

Visualization of the embryos was impossible with X-ray images. The percentage of undamaged seeds was more than 87% in all lots and treatments. There were no treatment differences between the percentages of firm (dense) and stained endosperms for all the seed lots in the tetrazolium test. Within each treatment, submitted to the tetrazolium test, the

percentage of firm and totally stained endosperms was similar to that of the undamaged seeds (Table 1), demonstrating the efficiency of the method for evaluating endosperm integrity.

The impossibility of observing the *Xylopia aromatica* embryo in the X-ray test can be attributed to the seed characteristics, which according to Castellani et al. (2001), are albuminous, with a dappled black and white endosperm,

hard and oily, with a whitish color and a continuous type embryo showing little differentiation.

In general, the seeds with firm endosperms also had tissue which is totally or mainly (> 50%), stained. In 2% of the seeds, which stained less than 50% (lot 1 – 0.75% at 25 °C and 0.5% at 30 °C; lot 2 - 1% at 30 °C) or which did not stain the endosperm (lot 1 – 0.75% at 25 °C), this tissue was firm (Table 2).

Table 1. Comparison between the mean percentage of *Xylopia aromatica* seeds with firm and completely stained endosperm tissues (F.C.T.), observed in the tetrazolium test, with undamaged seeds (U.S.), observed in X-ray images, for each lot and treatment; and comparison between the observed means in each treatment for both variables. I.T. = imbibition temperature; T.C. = tetrazolium concentration.

I.T.	Treatment	Lot 1		Lot 2		Lot 3	
	T.C.	F.C.T.	U.S.*	F.C.T.	U.S.*	F.C.T.	U.S.*
25 °C	0.5%	88 a A	87 a B	94 a A	97 a A	98 a A	100 a A
	0.75%	94 a A	95 a A	99 a A	99 a A	95 a A	97 a A
	1.00%	95 a A	97 a A	99 a A	99 a A	96 a A	98 a A
30 °C	0.5%	93 a A	97 a A	94 a A	95 a A	96 a A	97 a A
	0.75%	93 a A	93 a A	97 a A	98 a A	96 a A	99 a A
	1.00%	95 a A	96 a A	91 a A	96 a A	99 a A	99 a A

*Means followed by the same small letters within the same lot and treatment do not differ significantly according to the T test. Means followed by the same capital letters within each column do not differ significantly according to the LSD test at the 5% probability level. * Data obtained before the tetrazolium test.

Table 2. Mean percentage of endosperm characteristics of *Xylopia aromatica* from seeds evaluated by the tetrazolium test for each treatment and lot.

Treatment		Stained			Solidity		
		100%	> 50%	< 50%	none	firm	soft
0.5%/25 °C	L1	88	3	5	4	91	9
	L2	94	4	0	2	98	2
	L3	98	2	0	0	100	0
0.75%/25 °C	L1	94	2	1	3	99	1
	L2	99	0	1	0	99	1
	L3	95	2	1	2	97	3
1.00%/25 °C	L1	95	2	2	1	98	2
	L2	99	1	0	0	100	0
	L3	96	4	0	0	100	0
0.5%/30 °C	L1	93	4	2	1	98	2
	L2	94	1	0	5	95	5
	L3	96	2	2	0	98	2
0.75%/30 °C	L1	93	2	0	5	95	5
	L2	97	1	0	2	98	0
	L3	96	3	1	0	99	1
1.00%/30 °C	L1	95	3	0	2	98	2
	L2	91	4	2	3	96	4
	L3	99	1	0	0	100	0

The development of the red staining in the endosperm of the *Xylopia aromatica* seeds indicates that it has living

tissue. In most cultivated species, where the endosperm is a dead tissue, there is an aleurone layer, responsible for the

production of hydrolytic enzymes, which will degrade the endosperm (Olsen et al., 1995). Similarly, seeds of native species, in which the endosperm is also a dead tissue, have an aleurone layer with the same function. On the other hand, when the endosperm cells are alive there is no clear distinction between the endosperm and the aleurone layer and the enzymes are probably produced and liberated by

the endosperm cells themselves (Buckeridge et al., 2000).

In the evaluation of the embryonic tissue firmness of lot 1, the lowest percentage was observed in the treatment of 0.5% at 30 °C, only different from treatments 0.75 and 1% at 25 °C (Table 3). In this seed lot, the concentration of 0.5% of tetrazolium salt gave the highest percentage of embryonic tissue staining independent of the temperature.

Table 3. Comparisons between the mean percentages of embryos of *Xylopia aromatica* seeds with firm tissues (F.T.) or with firm and stained tissues (F.C.T.), observed in the tetrazolium test, and the results of seedling emergence and the formation of normal seedlings from seeds treated or not with Promalin®. I.T. = imbibition temperature; T.C. = tetrazolium concentration.

Treatment		Lot 1		Lot 2		Lot 3	
I.T.	T.C.	F.T.	F.C.T.	F.T.	F.C.T.	F.T.	F.C.T.
25 °C	0.5%	75 ab	50 b	63 a	22 de	76 a	43 bc
	0.75%	80 a	32 c	66 a	20 e	73 ab	44 bc
	1.00%	83 a	30 c	69 a	25 de	60 b	41 bc
30 °C	0.5%	69 b	54 b	60 ab	51 b	71 ab	50 b
	0.75%	77 ab	31 c	74 a	29 de	72 ab	33 c
	1.00%	74 ab	32 c	63 a	22 de	70 ab	39 bc
Control emergence (%)		53 c	53 b	46 bc	46 bc	2 d	2 d
Promalin emergence (%)		80 a	80 a	67 a	67 a	70 ab	70 a
Control normal seedling (%)		45 c	45 b	42 c	42 bc	2 d	2 d
Promalin normal seedling (%)		20 d	20 c	35 c	35 cd	39 c	39 bc

Means followed by the same letters within the same column do not differ according to the LSD test at the 5% probability level.

No differences between the seed percentages with firm embryos were observed in lot 2. However, the firm and stained tissue evaluation of the embryos of this lot showed that a salt concentration of 0.5% at a temperature of 30 °C gave the best results. In lot 3, the lowest percentage of firm tissue was observed in the 1% treatment at 25 °C while the 0.5% salt treatment at 30 °C also gave the best result and only differed from the 0.75% treatment at 30 °C (Table 3).

Not all the stained embryos were firm and neither were all the uncolored embryos soft (Tables 3 and 4). All the lots had some seeds with a necrotic embryonic region (Figure 2B) or did not have the embryos identified even after various cuts and were therefore considered as inexistent. According to Lobo et al. (2007), the Annonaceae family has pollination problems, which can lead to seed formation without any embryo.

Embryos were not found in some seeds and only the embryonic cavity in the endosperm was observed (Table 4). These embryos could have been lost during the cutting of the seeds for the tetrazolium test since Castellani et al. (2001) have described how *Xylopia aromatica* embryos are 268 times smaller than the endosperm.

All the viable embryos were found in seeds with firm

endosperms but these were not always completely stained. All the lots also had viable embryos in seeds with more than 50% staining and in only one seed with less than 50% staining, belonging to lot 1.

Many factors influence the degree of staining and penetration by the tetrazolium salt into the tissues, making result interpretation and standardization difficult. The color intensity may be greater in tissues which are cut, damaged, attacked by fungi or insects, or which are deteriorating. General aspects of tissues, such as the morphology and the consistency, should also be considered during seed analysis (Piña-Rodrigues and Santos, 1988).

The seeds from all the lots showed higher percentages of seedling emergence when treated with Promalin®, principally those from lots 1 and 3. However, a higher emergence speed was only observed in lot 1 (Table 5).

The highest percentages of normal seedlings were observed in lots 1 and 2, for the control and in lots 2 and 3 treated with Promalin®. The normal seedlings development was faster in seeds treated with Promalin®, with the exception of lot 1. Seed dormancy was observed in lot 3 due to the very low percentages and speed of emergence and the normal

seedling formation from untreated seeds (Table 5).

Most of the abnormal seedlings came from seeds treated with Promalin®, indicating a toxic effect caused by this product in seeds which did not have significant dormancy (lots

1 and 2). However, the highest percentage of seedlings with an incomplete development was observed in treated lot 1. There is a possibility that the proportion of normal seedlings from this lot would increase if they completed their development (Table 5).

Table 4. Mean percentage of embryo characteristics of *Xylopia aromatica* seeds evaluated by the tetrazolium test for each treatment and lot.

Treatment		Stained		Firmness		Nonexistent	Not found
		yes	no	firm	soft		
0.5%/25 °C	L1	52	35	75	12	10	3
	L2	22	70	63	29	5	3
	L3	45	42	76	11	5	8
0.75%/25 °C	L1	33	63	80	16	3	1
	L2	21	74	66	29	3	2
	L3	47	44	73	18	4	5
1.00%/25 °C	L1	37	57	83	11	5	1
	L2	26	71	69	28	1	2
	L3	45	43	60	28	2	10
0.5%/30 °C	L1	59	25	69	15	9	7
	L2	62	22	60	24	10	6
	L3	56	34	71	19	3	7
0.75%/30 °C	L1	35	56	77	14	6	3
	L2	31	62	74	19	4	3
	L3	34	53	72	15	6	7
1.00%/30 °C	L1	39	51	74	16	5	5
	L2	23	64	63	24	10	3
	L3	40	51	70	21	4	5

Table 5. Mean percentages of seedling emergence (E), seedlings with normal (NS), abnormal (AS) and incomplete development (ID) and mean emergence speed (SE) and normal seedling development (NSD) for *Xylopia aromatica*, after previous imbibitions in water (control) and Promalin®, for the three lots studied.

Treatment		E	NS	AS	ID	SE	NSD
Lot 1	Control	53 c	45 a	1 bc	7 b	0.026 c	0.018 ab
	Promalin	80 a	20 b	27 a	33 a	0.049 a	0.022 a
Lot 2	Control	46 c	42 a	4 b	0 c	0.020 d	0.015 b
	Promalin	67 b	35 a	24 a	8 b	0.039 b	0.022 a
Lot 3	Control	2 d	2 c	0 c	0 c	0.006 e	0.006 c
	Promalin	70 ab	39 a	17 a	14 b	0.025 cd	0.017 ab

Means followed by the same letters within the same column do not differ according to the LSD test at the 5% probability level.

In all the lots, the tissue integrity observed in the X-ray images is always better than seedling emergence and the formation of normal seedlings (Table 6). *Jatropha curcas* L. (physic nut) also had a significant number of dead seeds and abnormal seedlings originating from seeds classified as undamaged, according to X-ray images (Pinto et al., 2009).

With the exception of the 0.5% treatment at 30 °C in

lot 1, the percentage of seeds with firm embryos did not differ from the percentage emergence of seeds treated with Promalin®. The viability of the seeds with firm, stained tissues from lot 1 in the 0.5% tetrazolium salt treatments at both temperatures, showed no differences with the emergence and formation of normal seedlings from untreated seeds (Table 3).

The viability of the seeds with firm, stained tissues from lot 2 for the 0.5% treatment at 30 °C did not differ from the seedling emergence and normal seedling formation of untreated seeds. The formation of normal seedlings from seeds treated with Promalin® only differed in the viability observed in the treatments 0.75% at 25 °C and 0.5% at 30 °C (Table 3).

Table 6. Comparison between the mean percentages of undamaged *Xylopiya aromatica* seeds (US), observed in X-ray images, treated or not with Promalin®, and the results of seedling emergence (E) and normal seedlings development (NSD).

Treatment		US	E	NSD
		(%)		
Lot 1	Control	99 a	53 b	45 b
	Promalin	100 a	80 b	20 c
Lot 2	Control	98 a	46 b	42 b
	Promalin	97 a	67 b	35 b
Lot 3	Control	97 a	2 b	2 b
	Promalin	98 a	70 b	39 c

Means followed by the same letters on the same line do not differ according to the LSD test at the 5% level of probability.

Matteucci et al. (1997) also studied the viability of *Xylopiya aromatica* seeds using the tetrazolium test (0.5% at 30 °C, for 24 hours) and observed that seeds taken from mature fruits showed a 37% staining of vital parts. However, germination was not observed either in the laboratory or the nursery.

Annona cherimola L. and *Annona muricata* L., incubated at 30 °C in a 1% tetrazolium solution for 24 hours, showed 80% and 69% of viable seeds, respectively. However, germination was only 8% in *Annona cherimola* and zero in *Annona muricata*, seeds 30 days after sowing (Lobo et al., 2007).

The emergence of seeds from lot 3, recently harvested and treated with Promalin®, was higher than the viability observed in the tetrazolium treatments. However, this viability showed no difference with the percentage formation of normal seedlings originating from these seeds (Table 3).

Conclusions

X-ray images permit the evaluation of existing damage in the endosperm of *Xylopiya aromatica* seeds.

The tetrazolium test is efficient for evaluating seed viability, principally for those seeds which are imbibed at

30 °C for 48 hours and later immersed in a 0.5% tetrazolium solution for 24 hours at 30 °C.

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