X-ray and tetrazolium tests for assessing papaya seed viability

Testes de raios-X e de tetrazólio para avaliação da viabilidade de sementes de mamoeiro

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Highlights

Rapid testing methods are needed to assess papaya seed quality.
Rapid and reliable results are critical for quality control.
X-ray imaging effectively reveals the internal structures of seeds.
The tetrazolium test provides information on papaya seed viability.

Abstract

The slow and uneven germination of papaya seeds highlights the need for rapid and reliable tests to evaluate seed quality. This study aimed to assess the efficiency of X-ray and tetrazolium (TZ) tests in evaluating the physiological potential of papaya seeds. Seeds from five lots of the Formosa group were used. For X-ray image acquisition, 200 seeds from each lot were mapped and scanned to obtain data on area, perimeter, degree of filling, median gray level, and density. The seeds were evaluated for germination percentage and speed, and seedling growth (total length, vigor and growth indices). Preliminary tests were conducted to refine the TZ test methodology, which was then applied to the different lots. Embryos were classified as viable, non-viable, or dead. The X-ray test made it possible to visualize internal structures and detect filled, malformed, and empty seeds. The TZ test enabled the classification of lots according to their physiological potential, yielding results consistent with those obtained in the germination and seedling vigor tests. To this end, seeds were preconditioned on moistened paper for 48 hours to facilitate embryo extraction, followed by immersion in a 0.1% tetrazolium solution for 5 hours at 40 °C for staining.
Key words: Image analysis. Radiography. Physiological quality. *Carica papaya* L.

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Resumo

Devido à germinação lenta e desuniforme das sementes de mamão, testes que forneçam informações rápidas e seguras sobre sua qualidade são relevantes. Objetivou-se avaliar a eficiência dos testes de raios-X e de tetrazólio para avaliação do potencial fisiológico destas sementes. Foram utilizadas sementes de cinco lotes do grupo Formosa. Para aquisição das imagens de raios-X, 200 sementes de cada lote foram mapeadas e radiografadas para se obter dados de área, perímetro, preenchimento, média de cinza e densidade. Estas mesmas sementes foram avaliadas quanto à porcentagem e velocidade de germinação, bem como ao crescimento de plântulas (comprimento total es índices de vigor e crescimento). Para o teste de tetrazólio, foram realizados pré-testes para adequação da metodologia que foi aplicada aos diferentes lotes e os embriões classificados em viáveis, não viáveis e mortos. A técnica de raios-X permitiu visualizar as estruturas internas e detectar sementes cheias, malformadas e vazias. Pelo teste de tetrazólio, foi possível a classificação dos lotes em níveis de potencial fisiológico semelhante à obtida nos demais testes aplicados. Para tanto, as sementes devem ser pré-condicionadas em papel umedecido por 48 h para remoção do embrião, utilizando-se solução de tetrazólio a 0,1% por 5 h, a 40 oC para coloração.

Palavras-chave: Análise de imagens. Radiografia. Qualidade fisiológica. *Carica papaya* L.

Introduction

Brazil has become one of the world's leading papaya producers in recent years, with the fruit ranking among the country's seven most exported (Associação Brasileira dos Produtores e Exportadores de Frutas e Derivados [Abrafrutas], 2024). Papaya propagation is primarily by seeds, whose physiological quality is essential for uniform seedling establishment and the prevention of economic losses, especially when high-value hybrids are used. However, seed propagation is often limited by post-harvest dormancy and slow, irregular germination (Dias et al., 2015), which hinder the production of vigorous, uniform seedlings (Khajjak et al., 2022). Additionally, malformed seeds are commonly found in seed lots and may not be completely eliminated during processing.

The physiological quality of papaya seeds intended for commercialization is typically assessed by germination testing under optimal environmental conditions, requiring 30 days to obtain results that indicate the maximum germination percentage of a lot (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009). However, to support lot management decisions in seed production companies, it is also important to employ complementary tests that provide rapid and reliable information on lot performance. In this context, the X-ray and tetrazolium (TZ) tests are both recommended by the International Seed Testing Association [ISTA] (2004) and the Rules for Seed Analysis (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009) for several species.

The X-ray test is a simple, rapid, and non-destructive technique that enables visualization of internal seed morphology, revealing imperfections, void spaces, and physical damage through radiographic images (Nogueira et al., 2024). These features can be directly related to germination

capacity, as observed in *Cucumis melo* L. (Medeiros et al., 2020a), *Cucumis sativus* L. (Gomes et al., 2013), pumpkin (Antonio et al., 2016), and *Solanum aethiopicum* L. seeds (Alves et al., 2018). This technique is particularly relevant for papaya seeds, which often exhibit internal malformations that can affect field performance. Nogueira et al. (2024) reported that even fully filled seeds produce both normal and abnormal seedlings, indicating that X-ray imaging alone may not reliably predict seed physiological potential.

The TZ test provides a rapid estimate of seed viability based on the color reaction of living tissues when exposed to a 2,3,5-triphenyl tetrazolium chloride solution. This reduction process is catalyzed by dehydrogenase enzymes active in respiration, producing the stable, non-diffusible red compound triphenyl formazan (França et al., 2020b). The test clearly distinguishes viable living tissues from their non-viable counterparts, which remain unstained due to the absence of respiratory activity (França et al., 2020b). Its main advantage lies in its ability to generate results within a few hours, making it particularly valuable for seed quality control programs.

The efficiency of the TZ test depends on species-specific protocols defining optimal conditions for preconditioning, preparation, staining, and interpretation (Silva et al., 2020; Carvalho et al., 2018).

For example, a concentration of 0.075% is recommended for soybean (França & Krzyzanowski, 2020a), cotton (Von Pinho et al., 2020), and common bean seeds (Krzyzanowski et al., 2020b), whereas 0.1% is commonly used for coffee (Zonta et al.,

2009) and sunflower (Silva et al., 2020), and 0.5% for coriander seeds (Silva et al., 2021). For papaya seeds, Carvalho et al. (2018) suggested staining using longitudinally cut seeds in a 0.1% tetrazolium solution for 9 hours or a 1% solution for 6 hours. However, the longitudinal cut prevents a complete evaluation of embryonic tissues, and the method was validated using only a single seed lot. Therefore, further refinement and validation of the test are needed across different lots, correlating viability results with other physiological quality indicators.

Accordingly, the present study aimed to evaluate the efficiency of the X-ray and tetrazolium tests for assessing papaya seed viability.

Materials and Methods

The experiment was conducted at the Seed Research Laboratory of the Department of Agronomy, Federal University of Viçosa. Five papaya seed lots (Formosa group, cv. T2) were evaluated using the following tests:

Moisture content - determined using two replicates of 25 seeds, dried in an oven at 105 °C for 24 h (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009). Results were expressed as a percentage (wet basis).

X-ray test - eight replicates of 25 seeds were fixed onto adhesive paper, mapped, and labeled to allow correspondence with subsequent germination test results. Radiographic images were obtained using a Faxitron MX-20 cabinet X-ray system (Faxitron X-ray Corp., Wheeling, IL, USA), operated at 23 kV for 15 seconds at a 22- cm focal distance. Image contrast was calibrated

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to optimize seed visibility relative to the background (width × center: 2930 × 3830). Images were saved as TIFF (Tagged Image File Format) files and analyzed using ImageJ software and the IJCropSeed tool (Medeiros et al., 2020b) to obtain the following physical attributes: *area* – internal seed area (mm²); *perimeter* – length of the seed's outer boundary (mm); *filling* (%) – ratio between filled and empty spaces within the seed; *median gray value (gray·mm⁻¹)* – median gray level within the selection; and *relative density (gray·mm⁻¹)* – median gray level within the selection.

Germination - following the X-ray test, eight replicates of 25 seeds were sown according to the mapped sequence between sheets of Germitest paper sheets arranged in rolls, moistened with distilled water at 2.5 times the dry paper weight. The rolls were maintained in a BOD incubator at 20–30 °C under an 8-hour photoperiod (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009). The following parameters were evaluated: *Primary root protrusion timing* – daily counts of seeds with a primary root ≥ 2 mm long for 30 days, expressed as a percentage; *Primary root protrusion rate* – calculated from daily counts according to Maguire (1962); *Germination* – percentage of normal seedlings recorded at 15 and 30 days after sowing (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009); *First germination count* – percentage of normal seedlings at 15 days after the onset of the germination test (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009).

Seedling growth - eight replicates of ten seeds were placed longitudinally along the upper third of Germitest paper sheets, rolled,

and incubated at 20–30 °C (Krzyzanowski et al., 2020a). Assessments were performed 20 days after sowing. Photographs were taken with a Nikon Coolpix P510 digital camera and analyzed in ImageJ software using a reference image with a graduated ruler. Calibration was performed by drawing a 10 mm horizontal line corresponding to the ruler scale (*set scale* function), and seedling length was measured with the *Segmented line* tool. Growth and vigor indices were calculated using the Seedcalc package in R software.

Tetrazolium test - preliminary tests were conducted to define the optimal preconditioning, preparation, and staining procedures. Seeds were preconditioned for 16, 24, and 48 hours on paper towels moistened with distilled water (2.5× the dry paper weight) to facilitate seed coat removal, sectioning, and embryo extraction.

Embryos were extracted using a scalpel to make superficial cuts and remove the seed coat and endosperm. The embryos were then immersed in 2,3,5-triphenyl tetrazolium chloride solution at concentrations of 0.075% and 0.1% for 3, 5, and 7 hours at 40 °C in the dark.

Based on these preliminary results, the TZ test was applied to all five lots using the most suitable conditions, with four replicates of 50 seeds. After staining, seeds were rinsed in running water and kept hydrated to establish viability interpretation criteria for each lot.

Experimental design and statistical analysis - a completely randomized design with four replicates was used. Residual normality was evaluated using the Shapiro–Wilk test and homogeneity of variances with the Bartlett test. Data from each test were

subjected to analysis of variance (ANOVA), and lot means were compared using Tukey's test at a 5% probability level. Pearson's correlation analysis was performed between the results of the initial quality characterization and TZ tests. All statistical analyses were performed in R software version 4.3.3 (R Core Team [R], 2025).

Results and Discussion

Papaya seed moisture content ranged from 9.5% to 11.5% (Table 1), within the acceptable range for consistent results in both physiological potential assessments (Marcos, 2015) and X-ray testing, since seed moisture influences tissue optical density (Simak, 1991).

Table 1
Moisture content (MC), root protrusion (RP), root emergence speed index (RESI), germination (G), first germination count (FGC), total seedling length (TSL), growth index (GI), and vigor index (VI) of five papaya seed lots

Lot	MC (%)	G (%)	FGC (%)	RP (%)	RESI (index)	TSL (cm.seedling^{-1})	GI (index)	VI (index)
1	11.5	62 c	27 c	96 a	2.3 cd	2.7 b	95.9 d	306.8 c
2	9.5	70 bc	43 b	82 b	2.0 d	3.4 ab	129.3 b	324.8 b
3	10.8	74 b	12 d	95 a	2.5 c	3.3 ab	116.5 c	328.4 b
4	9.9	82 a	75 a	96 a	3.6 a	3.4 a	151.6 a	357.1 a
5	9.7	79 ab	1 d	98 a	2.7 b	3.3 ab	131.8 b	360.3 a
CV (%)	-	11.1	7.9	3.6	6.4	11.9	4.0	3.0

Means followed by the same letters within columns do not differ significantly according to Tukey's test (p < 0.05). CV: coefficient of variation.

Significant differences in physiological potential were observed among seed lots (Table 1). Germination results indicated that Lots 4 and 5 outperformed Lot 1, which did not differ significantly from Lot 2. All lots met the 60% minimum germination standard established for papaya seeds by Ordinance No. 538/2022 of the Brazilian Ministry of Agriculture, Livestock, and Supply (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2022).

The percentage of normal seedlings at seven days (first count) was lowest for Lots 3 and 5, followed by Lot 1, which was lower than Lot 2, whereas Lot 4 exhibited significantly higher values compared to the other lots. However, root protrusion data showed that only Lot 2 was inferior to the others, which displayed high percentages (95-98%. These findings indicate that a large proportion of Lot 5, 3, and 1 seeds initiated root protrusion but failed to sustain development, resulting in lower percentages of normal seedlings at the first count. The primary root emergence index confirmed Lot 4 as the most vigorous, while Lot 2 exhibited the lowest rate, not differing significantly from Lot 1. Lot 2 also displayed the lowest root protrusion overall.

Seedling growth results (Table 1) showed that Lot 4 produced longer seedlings than Lots 1 and 5. Similarly, Lot 4 had the highest growth index, while Lot 1 obtained the lowest values for both growth and vigor, with Lots 4 and 5 performing best. Overall, Lot 4 consistently ranked among the highest-performing lots, contrasting sharply with Lot 1 in total seedling length, growth, and vigor indices. Seeds with higher physiological potential tend to form more developed seedlings due to greater efficiency in cellular repair, reserve mobilization, and tissue synthesis during germination (Krzyzanowski et al., 2020a).

Studies have demonstrated the reliability of seedling length tests and growth and vigor indices in assessing seed physiological potential in several species, including lentil (Limão et al., 2023), chickpea (Araújo et al., 2021), maize (Andriazzi et al., 2023), and drumstick tree (Pereira et al., 2020).

As illustrated in Figure 1, X-ray images clearly revealed the internal structures of papaya seeds, allowing easy distinction between full (Figure 1A) and empty seeds (Figure 1D). These results demonstrate the potential of the X-ray test in identifying the developmental stage of internal seed tissues.
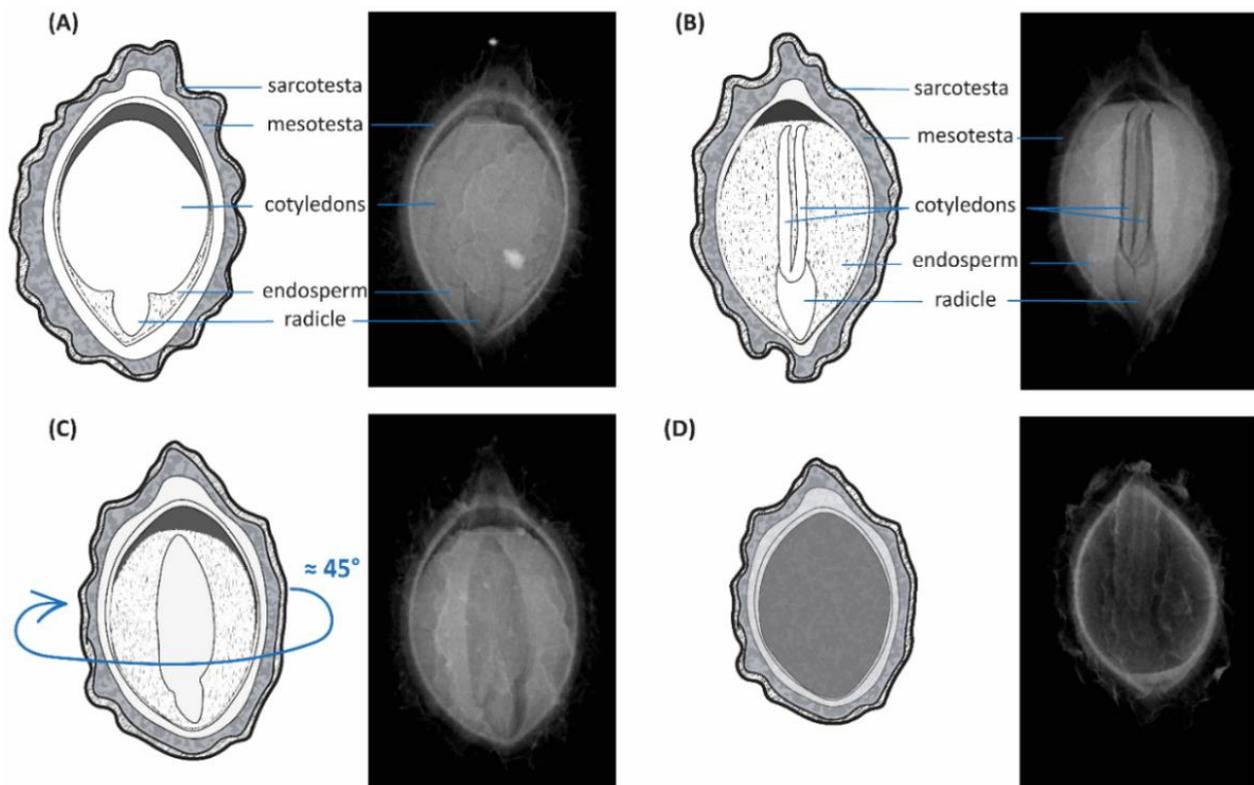

Figure 1. Radiographic (X-ray) images of papaya seeds showing internal structures: (A) frontal view, (B) lateral view, (C) ≈ 45° view of a full and well-formed seed, and (D) empty seed.

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X-ray testing of papaya seeds following sarcotesta removal allowed classification into four categories: empty, full, damaged or malformed, and dead or dormant, which were correlated with seedling performance in the germination test (Severiano et al., 2018). This information is critical for seed lot quality control, since external appearance alone cannot identify these categories.

In the present study, quantitative image metrics were used to enhance the analysis of X-ray test results. However, one of the challenges encountered was standardizing seed positioning to ensure uniform embryo orientation in the generated images. Variation in positioning influences the acquisition of metrics such as relative density, integrated density, median gray value, and filling, which are commonly used to relate seed physiological quality to X-ray images (Medeiros et al., 2020b). Higher density in *Senna macranthera* (Araújo et al., 2023) and melon seeds (Medeiros et al., 2020b) has been linked to greater physiological potential, but this relationship was not observed in our study (Figure 2). Differences in cotyledon angles (Figures 1B and 1C) likely contributed to variations in median gray values, since endosperm thickness and inter-cotyledon spaces affected image brightness.
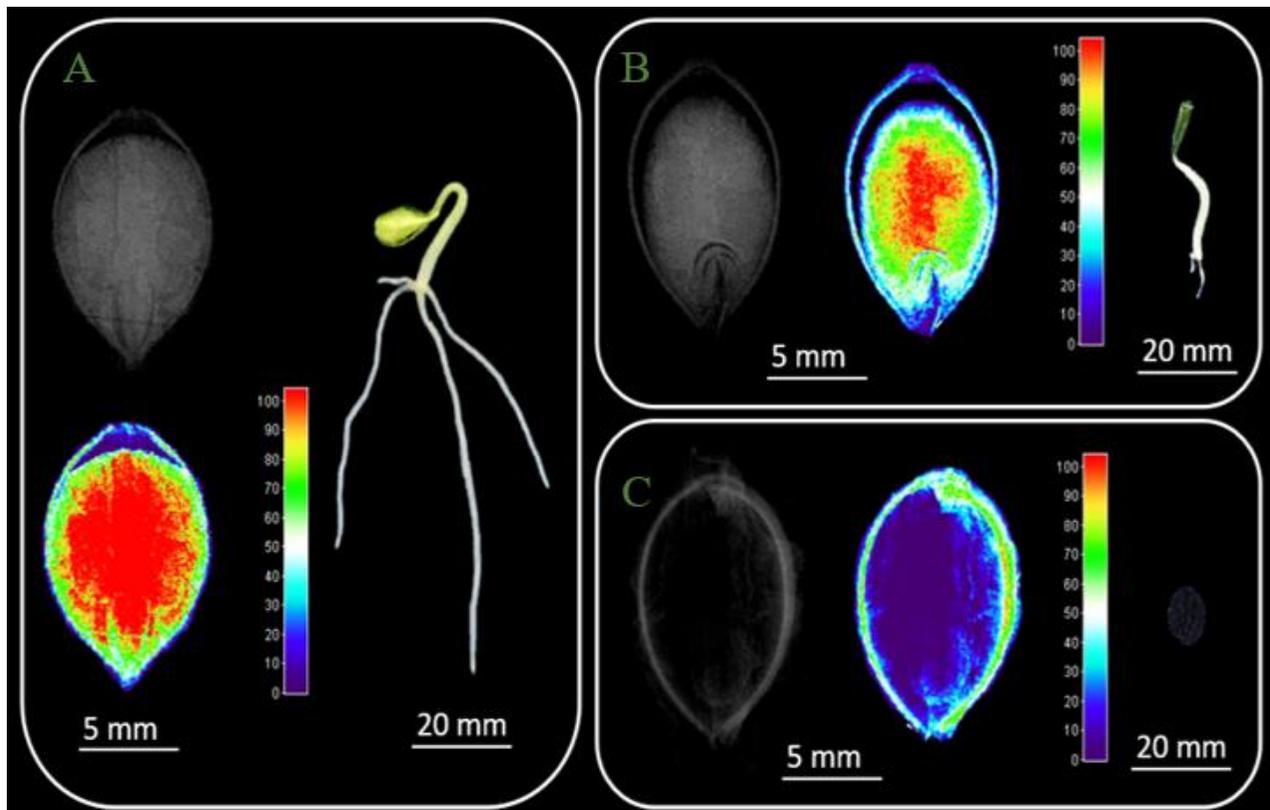

Figure 2. Radiographic (X-ray) image of the seed, heat map based on relative seed density, and image of the seedling obtained from each papaya seed (A – with a well-defined and fully developed embryo; B – with embryo damage; C – empty seed).

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Figure 3 shows that the area (Figure 3A) and perimeter (Figure 3B) values obtained from X-ray images were highest for Lot 1, followed by Lots 2, 3, and 4, with the lowest values observed in Lot 5. However, these traits were not related to physiological potential, given that the larger Lot 1 seeds did not perform better (Table 1). Previous studies have demonstrated that seed size is not always a reliable predictor of physiological potential, which includes both germination and vigor (Marcos et al., 2000).
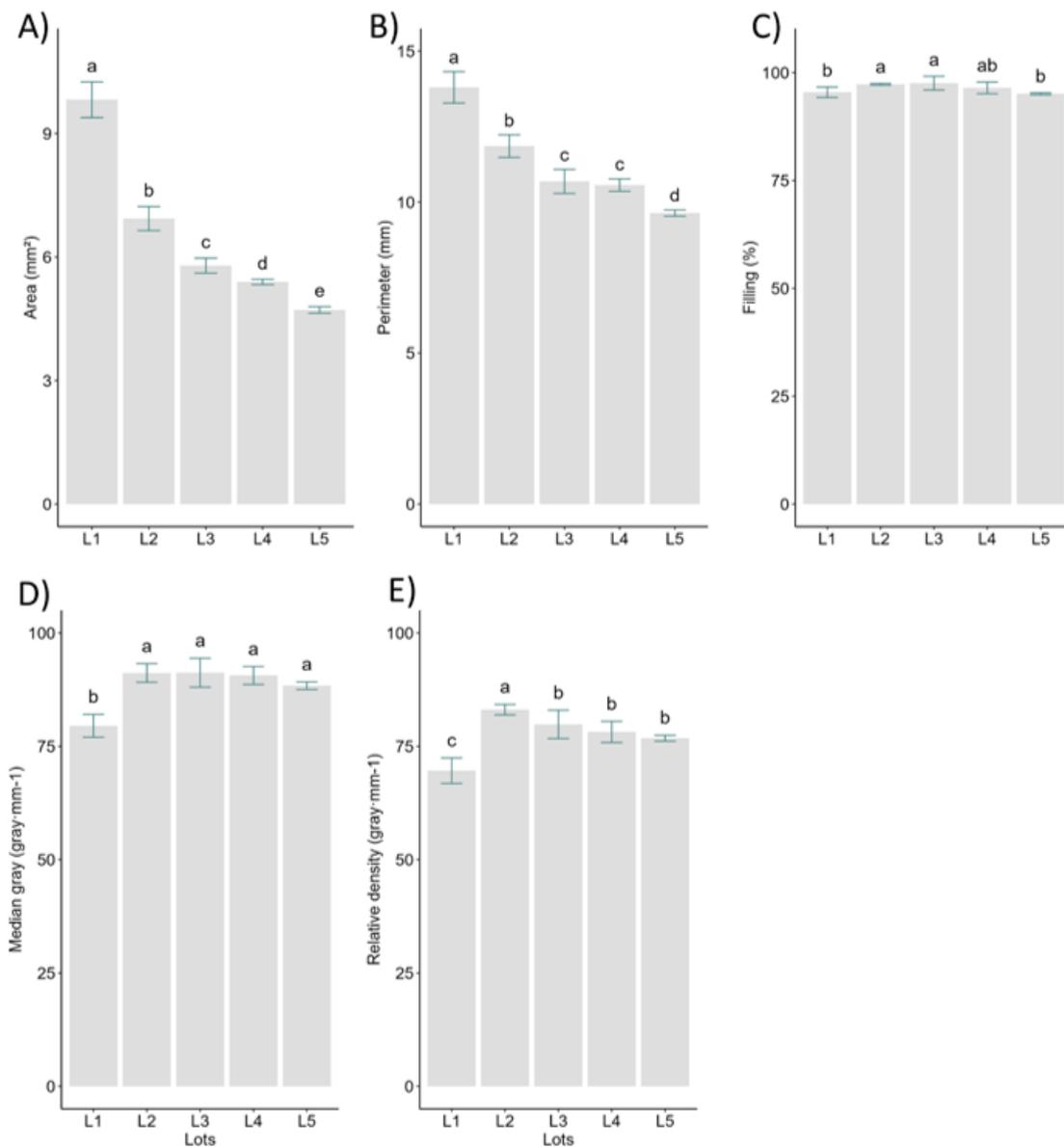

Figure 3. Area (A), perimeter (B), filling (C), median gray value (D), and relative density (E) for five papaya seed lots. Identical letters do not differ according to Tukey's test (p < 0.05). Bars represent the standard deviation.

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For the filling variable (Figure 3C), Lot 2 and 3 seeds were superior to those from Lots 1 and 5. Notably, Lot 1, which was among those with the poorest physiological potential (Table 1), also exhibited the lowest ash (Figure 3D) and relative density (Figure 3E), corroborating a pioneering study showing that sorghum seeds with lower density and ash content exhibit reduced physiological potential (Maranville & Clegg, 1977).

The X-ray test effectively distinguished empty and damaged papaya seeds (Figure 2) from filled seeds with well-formed embryos, which is a major advantage since these distinctions cannot be made through visual inspection alone. However, this technique did not provide enough information to infer seed physiological potential.

By contrast, the TZ test is a promising alternative for evaluating internal structures and their relationship to the physiological potential of papaya seeds, provided that the methodology is properly defined. Thus, preliminary tests were conducted to refine procedures for seed preconditioning, preparation, staining, and result interpretation. Among the preconditioning periods tested, seed imbibition for 16 or 24 hours on moistened paper towels did not ensure adequate tissue hydration. The tegument and endosperm remained rigid, making embryo removal difficult and often resulting in damage. Conversely, preconditioning for 48 hours provided the best results, since it facilitated cutting the tegument and endosperm with a scalpel, allowing embryo removal from the seeds without causing damage. Carvalho et al. (2018) reported satisfactory results using only 2 hours of preconditioning prior to longitudinal cutting to stain internal tissues (endosperm and embryo). However, this approach does not permit detailed embryo assessment or clear visualization of deteriorated or dead tissues in the cotyledons and embryonic axis. In the present study, complete embryo extraction was essential for accurate viability assessment, which required 48 hours of imbibition (preconditioning). This extended period was necessary because the internal tissues of the seeds were still rigid after two hours of hydration. Furthermore, papaya seeds typically require about 30 days to germinate, implying the need for longer imbibition to adequately activate the enzymatic system, especially dehydrogenase enzymes involved in mitochondrial respiration (França et al., 2020b), thereby improving staining efficiency in the viable tissues. Proper seed preconditioning ensures sufficient tissue hydration, facilitating seed preparation and enhancing tetrazolium penetration, thereby simultaneously activating pre-germinative metabolism, particularly the dehydrogenase enzymes (Von Pinho et al., 2020).

After preconditioning and preparation, the extracted embryos were immersed in tetrazolium solutions at two concentrations. At 0.075%, staining was weak and slow, most embryos appeared pale pink or almost white (Figure 4), making it difficult to classify tissue viability. By contrast, the 0.1% concentration produced a more distinct coloration, allowing clear differentiation between viable (bright pink), deteriorated (bright red), and dead (white) tissues with no respiratory activity (França et al., 2020b). Thus, the 0.1% tetrazolium solution was considered optimal for papaya seed evaluation and interpretation. Lower tetrazolium concentrations (around 0.075%)

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are typically used for soybean (França & Krzyzanowski, 2020a), cotton (Von Pinho et al., 2020), and common bean (Krzyzanowski et al., 2020b), whereas higher concentrations (around 0.1%) are recommended for coffee (Zonta et al., 2009), sunflower (Silva et al., 2020), and papaya (Carvalho et al., 2018). For forage grasses, Custódio and Aguiar (2020) suggest concentrations between 0.1 and 0.3%.
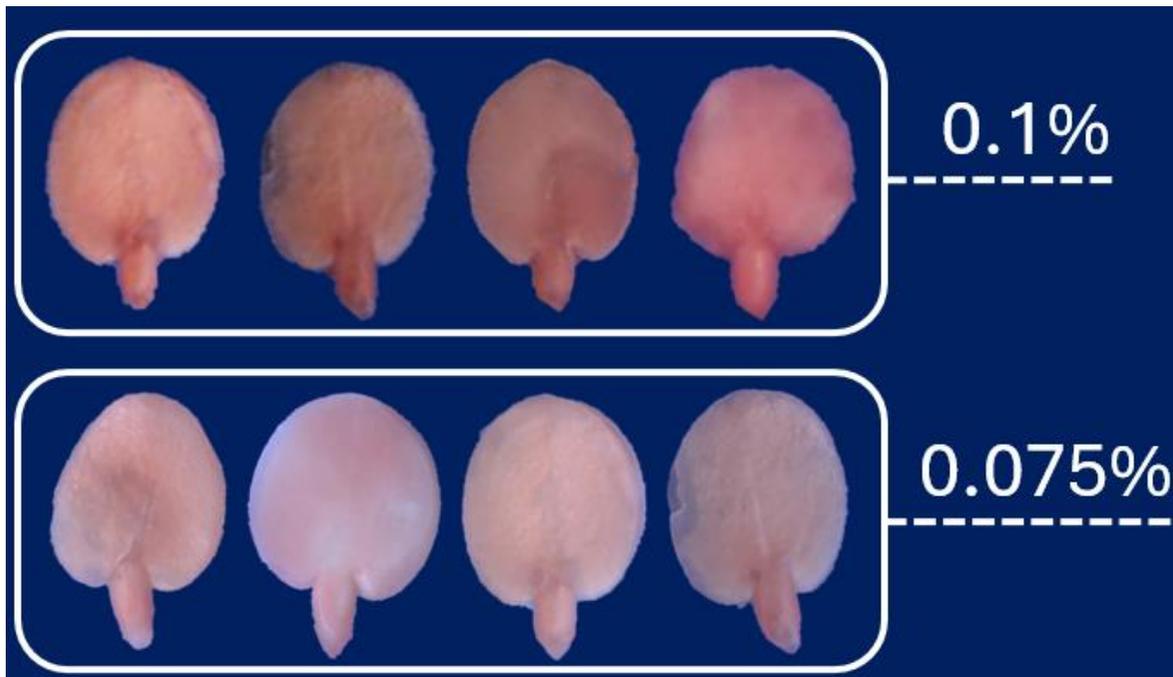

Figure 4. Papaya embryo staining in 0.1% and 0.075% tetrazolium solution for five hours at 40°C.

The staining time of 3 h was insufficient at both 0.1% and 0.075% concentrations, whereas 7 h was excessive, producing overly intense red coloration that hindered differentiation between viable and deteriorated tissues. A 5-hour stain period provided the clearest results, allowing reliable identification of color variations within the embryo (Figure 4). It is important to underscore that staining duration is generally inversely proportional to tetrazolium concentration. For instance, Carvalho et al. (2018) tested concentrations of 0.05%, 0.1%, 0.5%, and 1% for 3, 6, and 9 h, observing that 3 h was inadequate at all levels, whereas 6 h at 1% and 9 h at 0.1% produced viability estimates comparable to germination test results. However, these authors used only one longitudinally sectioned seed lot, which enabled visualization of the endosperm but hindered full evaluation of embryo color (cotyledons + hypocotyl–radicle axis) and the detection of localized tissue damage.

Based on TZ test results, seeds were preconditioned on moistened paper for 48 hours to facilitate embryo extraction, followed by staining in a 0.1% tetrazolium solution for 5 hours. Three viability classes were established according to tissue staining intensity and the presence, location, and extent of lesions (Figure 5). Classification criteria emphasized the integrity of tissues essential for seedling development. According to França and Krzyzanowski (2020a), damaged or dead areas may be present, provided they do not affect the root meristem or the vascular tissues located in the lower middle third of the cotyledons, near their insertion into the embryonic axis. This region is crucial for translocating reserves from the cotyledons to the developing axis during germination. Lesions in this region can compromise seed viability (França & Krzyzanowski, 2020a).

Figure 5. Papaya seed embryo staining pattern in the tetrazolium test for each class: class 1 (viable seeds), class 2 (non-viable seeds), and class 3 (dead seeds).

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The following classifications were established: Class 1 – viable seeds: embryos with firm, turgid tissues showing pink to bright red coloration with no structural damage (Figure 5a). Small areas with red or milky-white lesions may be present, provided they are confined to the edges of the cotyledons and do not exceed 50% of their total area. The integrity of vital regions is key for normal germination. Class 2 – non-viable seeds: embryos with cotyledons, areas at the cotyledon-axis junction and in the embryonic axis showing more than 50% milky-white or bright red tissue (Figure 5b). Class 3 – dead seeds: embryos with flaccid milky-white or bright red tissues (Figure 5c).

The TZ test viability results (Figure 6) indicated a higher percentage of Class 1 (viable) seeds in Lot 4, which did not differ significantly from Lot 5. Lot 1 exhibited the lowest viability, with a higher percentage of nonviable and dead seeds compared to the other lots. Viability results were consistent with germination data (Table 1) both classifying the lots similarly in terms of physiological potential, a finding supported by correlation analysis (Figure 7).
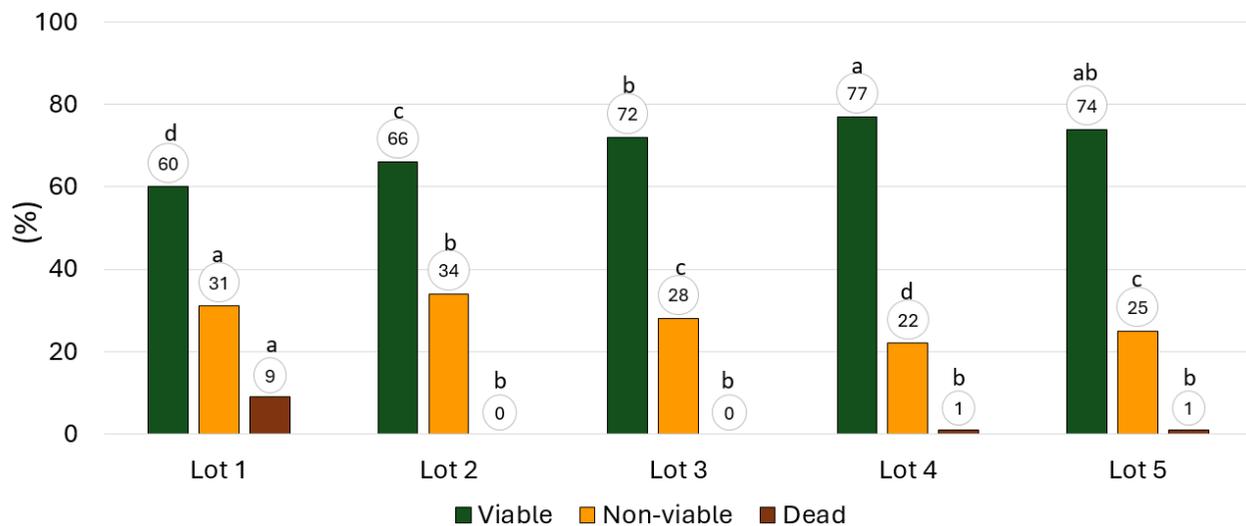

Figure 6. Percentage of papaya seeds in each viability class according to the tetrazolium test for five seed lots:
Class 1 (viable seeds), Class 2 (non-viable seeds), and Class 3 (dead seeds). Means followed by the same letters do not differ according to Tukey's test ($p < 0.05$). Bars on each column represent the standard deviation for each lot.
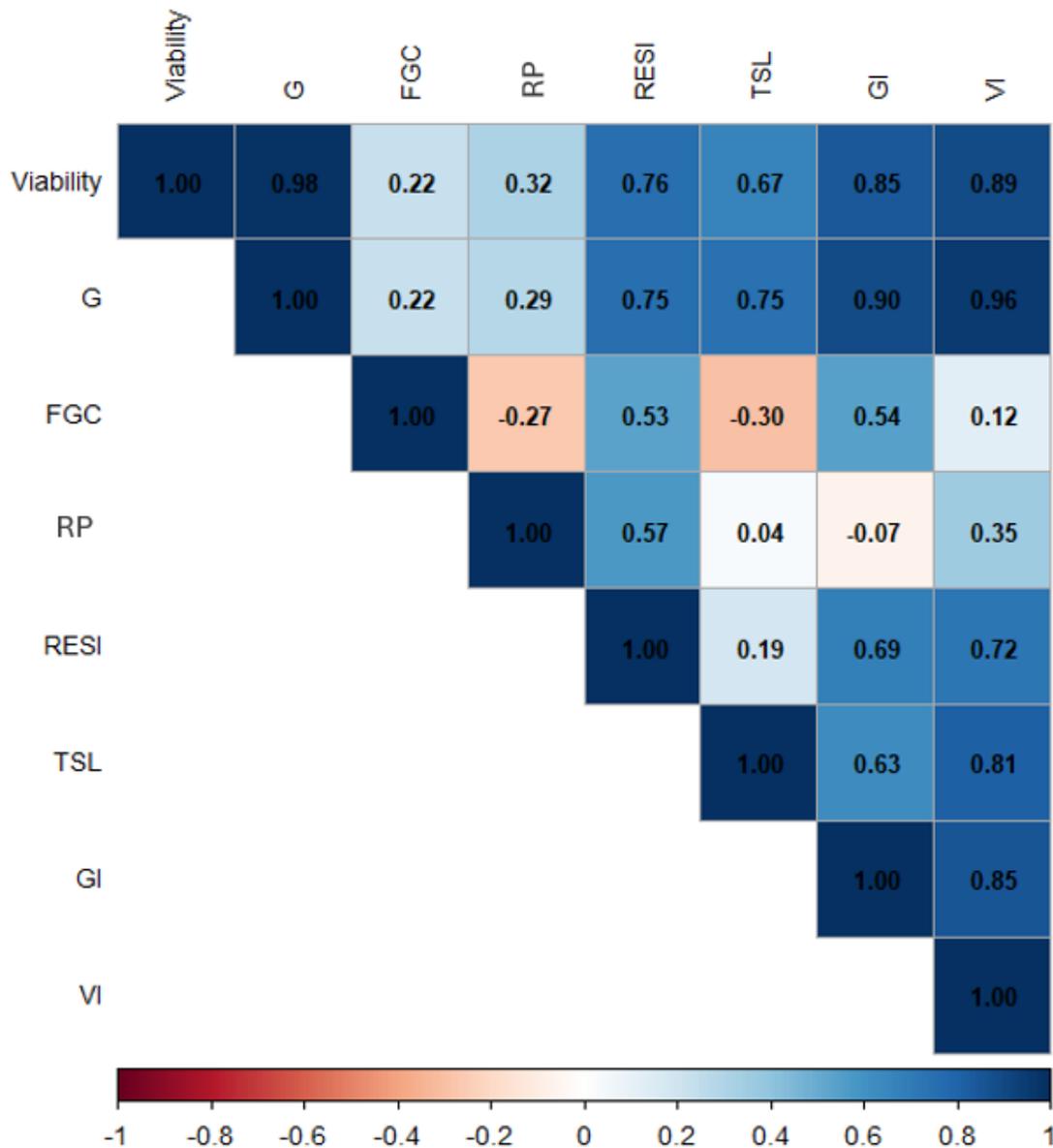
Figure 7. Pearson's simple correlation coefficients (r) estimated between viability determined by the tetrazolium test, germination (G), first germination count (FGC), root protrusion (RP), root emergence speed index (RESI), total seedling length (TSL), growth index (GI), and vigor index (VI) in seeds from five papaya seed lots.

The TZ test (Figure 6) revealed greater viability in Lot 4 compared with Lots 1, 2, and 3, a classification consistent with that obtained from germination tests, the Index of Seedling Vigor (IVER), seedling length, and growth and vigor indices (Table 1). These results are corroborated by Pearson's correlation analysis (Figure 7), which confirmed positive associations between viability, germination, and seedling length, reinforcing the findings presented in Table 1 and Figure 6.

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Furthermore, no significant difference was observed between Lots 4 and 5 in either the TZ or germination tests. Overall, both methods enabled consistent identification of the best and worst performing lots, with minor variations. Lot 4 consistently ranked among those with the highest physiological potential, while Lot 1 was among those of lowest quality. Viability and germination percentages were also numerically similar, differing by only 2% to 5%. According to França and Krzyzanowski (2020a), viability and germination results for soybean seeds should differ by no more than 5%. Given that papaya seed germination requires 30 days (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009), the TZ test is an important alternative for viability assessment in quality control programs. Rapid and efficient seed analysis techniques are valuable tools for papaya seed producers to help determine the lot destination at each harvest and define post-harvest management, storage, and commercialization strategies. This includes discarding low-performance lots to avoid unnecessary processing and storage costs. It is important to note that the TZ test is already used as a reference method for species with long germination times, such as coffee (30 days), brachiaria (21 days), and guinea grass (28 days) (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009).

Conclusions

The X-ray test enables the effective visualization of internal papaya seed structures, making it possible to identify full, malformed, and empty seeds, and well-developed embryos. However, there is no direct relationship with seed physiological potential.

The tetrazolium test proved efficient for assessing papaya seed viability, providing results consistent with germination tests. For accurate diagnosis, seeds should be preconditioned on moistened paper for 48 hours to facilitate embryo extraction, followed by immersion in a 0.1% tetrazolium solution at 40°C for 5 hours to ensure adequate staining.

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