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Viability and vigor of *Moringa oleifera* Lam. seeds using the tetrazolium test

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Abstract

To evaluate the physiological potential of the seeds, the tetrazolium test would be an interesting alternative, as it is a quick test that provides a detailed analysis of viability and vigor. Therefore, it is necessary to define pre-conditioning conditions, such as temperature and duration of soaking, concentration of the tetrazolium solution, and method of exposing the seeds to staining. *Moringa oleifera* Lam., belonging to the family Moringaceae, is widely distributed in tropical countries and easily adapted to semi-arid conditions, possessing medicinal value, as forage, condiment, in the cosmetics industry, melliferous, fuel, and in water purification treatment through the coagulant solution made from seeds. Based on the above, the objectives of the present work were to establish the most appropriate procedure for conducting the tetrazolium test to evaluate the viability and vigor of *M. oleifera* seeds. Only the cotyledons containing the embryo were placed in 50 mL plastic cups and immersed in a solution of 2,3,5-triphenyl chloride tetrazolium salt at concentrations of 0.05; 0.075 and 0.1% for staining periods of 1, 2, and 3 hours in a Biochemical Oxygen Demand chamber at 30 ºC in the dark. The seeds were evaluated one by one for tissue condition (firm or flaccid) and seed color, and by the location and size of the lesions on the cotyledon and embryonic axis. For the standardization of the tetrazolium test, the Completely Randomized Design was used in a 3x3+1 factorial scheme (three concentrations of tetrazolium solution x three staining periods + control = germination test) with four repetitions of 25 seeds. *M. oleifera* seeds were found to have a high germination rate (100%), there was no significant difference between the control and the percentage of viable seeds (TZ) for the 2 and 3 hours staining periods, immersed in tetrazolium solution at concentrations of 0.075 (96%) and 0.05% (99%), respectively. The most appropriate procedure for conducting the tetrazolium test to assess the viability and vigor of *M. oleifera* seeds is to use a concentration of 0.05% for 3 hours of salt immersion.

Keywords: classes of viability. classification of vigor. Moringaceae. semiarid conditions.
Abbreviations: ANOVA_Analysis of Variance; B.O.D._Biochemical Oxygen Demand; DIC_Completely Randomized experimental Design; TZ_Percentage of viable seeds.

Introduction

To evaluate the physiological potential of seeds, the tetrazolium test can be an interesting alternative, because it is a quick test that provides a detailed analysis of viability and vigor (Paraíso et al., 2019). In general, te test can be completed in less than 24 hours, enabling to identify the presence, location, and nature of any damage to the seeds (Santos et al., 2019).

In the tetrazolium test, the dehydrogenase enzymes act in cellular respiration, producing a red substance, stable and non-diffusible. This compound is called triphenylformazan, is formed from the reduction of 2,3,5-triphenyl tetrazolium chloride. As a result of this reaction, the living parts of the seed are dyed red, while the dead retain their original color, allowing to distinguish tissues by their condition (França-Neto; Krzyzanowski, 2019).

To perform the tetrazolium test, it is necessary to define preconditioning conditions, as temperature and duration of soaking, concentration of the tetrazolium solution and method of exposing the seeds to staining (Mercado et al., 2020; Pereira et al., 2020). There is no specific methodology in the Rules for Seed Analysis that recommends the tetrazolium test for *Moringa oleifera* (Brasil, 2009).

Germination is one of the most sensitive phases in the life cycle of plants. Carvalho and Nakagawa (2012) explained that it begins with tissue hydration, imbibition, responsible for triggering the resumption of metabolic reactions (catabolism), activating the degradation of reserves. These compounds are stored during the phase of intense metabolism (anabolism), when the seed was still connected to the mother plant with the use of oxygen (respiration), for the production of chemical energy responsible for the growth of the embryonic axis, culminating the rupture of the cover and primary root protrusion (Pastorini et al., 2016).

Moringa oleifera Lam. belongs to the family Moringaceae, is widely distributed in tropical countries, and for being easily adaptable to semi-arid conditions, has been excelling in scientific research (Medeiros et al., 2019). The species also has multiple uses, mainly for its medicinal value, as forage, condiment, in the cosmetics industry, melliferous, fuel, and in water purification treatment through the coagulant solution made from seeds (Noronha et al., 2019).
Based on the above, this work aimed to establish the most appropriate procedure for conducting the tetrazolium test to evaluate the viability and vigor of *M. oleifera* seeds.

Results and discussion

Standardization of the tetrazolium test

There was a significant difference in staining periods in *M. oleifera* (F = 128.09; p = 0.001). The lowest percentages of viable seeds were obtained by immersing the seeds in a tetrazolium solution for 1 hour (Table 1). These results were attributed to the difficulty in distinguishing the viability classes, due to the short penetration time of the salt in the seed tissues.
Likewise, there was a significant difference in the concentrations tested (F = 92.03; p = 0.001).
Shorter periods of seed exposure, whose coloring is generally less intense, to 2,3,5-triphenyl chloride tetrazolium salt makes it difficult to distinguish between living and dead tissue (Silva et al., 2013).
According to Table 1, only at the end of the period of 3 hours at a concentration of 0.05%, and 2 hours at a concentration of 0.075% (F = 9.94; p = 0.001), it was possible to observe the highest estimates of seed viability. Fabric color also allowed for easier visual assessment and class differentiation. This highlighted the need to develop standard tetrazolium procedure for *M*. *oleifera*.
Furthermore, there was a significant difference between the result of the germination test (control) and the average percentage of viable seeds (TZ) in each combination of the tetrazolium test (F = 9774.18; p = 0.001).
According to Cunha and Gomes (2015), the species *Erythrina velutinana*, exhibited coloration that helped in better distinguishing viable and non-viable seeds in 0.05% tetrazolium solution for 3 hours. For the different concentrations, there was a reduction in the average seed viability as the concentration of the tetrazolium solution increased.
Furthermore, the 0.05% solution is one of the most viable concentrations for the viability of *Handroanthus impetiginosus* in relation to the germination test (Nascimento, 2017). It is also more economical because it requires less 2,3,5-triphenyl chloride tetrazolium salt and can perform several tests with small quantities.
Some plant species can show complications during differentiation, if the endosperm layer is extremely thick. The endosperm tick layer precludes the tetrazolium salt to enter in the seed and the tissue shows a milky white (dead) appearance. Meanwhile, if the layer is thin, the action of the salt on the embryo will make the coloring more intense, resulting in the appearance of deteriorated tissue (Silva et al., 2016). According to Souza et al. (2017) these data are necessary for correct results about seed quality.

Viability by the tetrazolium test

It was found that *M*. *oleifera* seeds had a high germination rate (100%), with no significant difference between the control and TZ for the staining periods of 2 and 3 hours, immersed in tetrazolium solution at concentrations of 0.075 (96%) and 0.05% (99%), respectively (Table 2). Therefore, these conditions provided a better interpretation of the protocol, with the distinction of colored tissues and differentiation between them, associated with the reduction of immersion time or salt concentration.
These results corroborate with those obtained by Carvalho et al. (2017), studying the vigor of *Libidibia ferrea* seeds, where they observed that at the 0.05% concentration there was no difference, compared to the control. The same goes for *Tabebuia roseoalba* seeds at a concentration of 0.05%, showed effectiveness in assessing seed viability (Brito et al., 2020).
According to Marcos-Filho (2015), the choice of the appropriate methodology for the tetrazolium test should be based on the ease of differentiating viable and non-viable tissues, and on the ability to separate batches with different physiological qualities.
On the other hand, studies involving procedure, pre-conditioning, staining and viability classes, standardizing the tetrazolium test for *M*. *oleifera* or other Moringaceae species were not found.
Thus, it was inferred that the tetrazolium test, at a concentration of 0.05% of the salt for 3 hours was the most effective in assessing viability for *M*. *oleifera* seeds.
The percentage of viable seeds (TZ) was 95, 94 and 97%, for seeds harvested in 2020, in the months of August, September and December, respectively, and of 99 and 100%, for seeds harvested in 2021, in the months of April and June, respectively (Table 3).
Seeds harvested in 2020, and freshly harvested (2021), show good viability, good vigor and without any limitation regarding mechanical damage, stink bugs and humidity.
For adequate staining of many forest species high amounts of tetrazolium solution are required (Nogueira et al., 2014). However, this was not applied to the species that we studied here, which obtained the best results in the low concentrations.

Materials and methods

The trial was conducted in the Plant Propagation Laboratory, of the Campus of Engineering and Agricultural Sciences (CECA), from the Federal University of Alagoas, Rio Largo, AL, Brasil.

Plant materials

To obtain seeds, fruit harvesting was done, from ten trees located in the experimental area of the CECA, located at 09º27´57´´S, 34º50´01´´W and at 127 meters of altitude. The climate is of type As, tropical wet coastal, according to Köppen's climate classification (Cardim, 2003).
The fruits were harvested with a telescopic extension pruner at the end of the ripening period, characterized by a dark brown coloration, before the spontaneous opening, and after that, kept in artificial shade (shelter from sun and rain) for a few days, to facilitate seed extraction (Agustini et al., 2015).

Table 1. Percentage of viable seeds of *M. oleifera*.

Concentration (%)	Time in contact with salt (hour)			
	1	2	3	Average
0.05	52Ab	60Bb	99Aa	70
0.075	43Bc	96Aa	72Bb	70
0.1	11Cb	31Ca	37Ca	26
Average	35	62	69	55

Means followed by the same lowercase letter do not differ significantly from each other at 5% probability, within each exposure time. Capital letters indicate the differences for the means between the concentrations of tetrazolium.

Table 2. Percentage of viable seeds (TZ) of *M. oleifera* compared to the result of the germination test (control).

Tetrazolium test	Control (%) = 100
	TZ (%)
0.05% 1 hour	48
0.05% 2 hours	40
0.05% 3 hours	1^{NS}
0.075% 1 hour	57
0.075% 2 hours	4^{NS}
0.075% 3 hours	28
0.1% 1 hour	89
0.1% 2 hours	69
0.1% 3 hours	63

^{NS}Not significant, by Dunnett's test, at 5% probability level.

Table 3. Percentage of viable seeds (TZ) of *M. oleifera* harvested in 2020, and recently harvested (2021), depending on the best condition of the tetrazolium test

Year	Month	TZ (%)
2020	August	95 b
	September	94 b
	December	97 ab
2021	April	99 a
	June	100 a

The means followed by the same letter, in the column, do not differ statistically from each other by the Tukey test at the 5% significance level.

The seeds were extracted and processed by hand, discarding the malformed ones, attacked by insects or fungi, and following, conditioned in glass jars and stored in a dry chamber at a temperature of ± 20 ºC and relative humidity between 50 and 55% until the experiments (Pereira et al., 2015).

Tetrazolium test

To facilitate the absorption of the tetrazolium solution, the seeds were pre-moistened between paper towels, as described for the germination test, and placed in a Biochemical Oxygen Demand (B.O.D.) chamber at 25 ºC for five hours. Then, the seeds were cut longitudinally with a n.º 22 scalpel blade through the distal half of the cotyledons, leaving the embryonic axis intact (Brasil, 2009).

Only the cotyledons containing the embryo were placed in 50 mL plastic cups, and immersed in a solution of 2,3,5-triphenyl tetrazolium chloride salt, at concentrations of 0.05, 0.075 and 0.1%, for the staining periods of 1, 2, and 3 hours, in a B.O.D. type chamber at 30 ºC in the dark. At the end of the staining period, the solution was discarded and the seeds were washed in running water and kept submerged in water until the time of evaluation.

Based on staining patterns and tissue health the seeds whose embryonic axis presented pink coloration were considered to be viable. The non-viable seeds were those with discolored embryonic axis or with yellow or very intense red coloration (França-Neto and Krzyzanowski, 2018).

In order to determine the vigor classification and to categorize the seeds into different viability classes, the best condition of the tetrazolium test was applied to seeds harvested in 2020, and freshly harvested seeds (2021).

The seeds were evaluated one by one for tissue condition (firm or flaccid) and seed color, and by the location and size of the lesions on the cotyledon and embryonic axis (Masullo et al., 2017).

Interpretation was performed under a six magnification loupe with fluorescent illumination. The results were expressed in percent viable seeds (TZ).

Experimental design and statistical analysis

For the standardization of the tetrazolium test, the Completely Randomized experimental Design (DIC), was used, in a 3x3+1 factorial scheme (three concentrations, of 0.05, 0.075 and 0.1% of the tetrazolium solution x three staining periods + control = germination test) with four replications of 25 seeds.

Data were submitted to Analysis of Variance (ANOVA), and the TZ means obtained by the tetrazolium test were compared by the Tukey test ($p \leq 5\%$). The comparison between the TZ averages, for each of the combinations in the tetrazolium test, with the results of the germination test (control) was performed by the Dunnett test ($p \leq 5\%$). The statistical software used was ASSISTAT 7.6 beta.

The viability evaluation by the tetrazolium test was performed using DIC with four replications of 50 seeds. The TZ data were subjected to ANOVA. The means were compared using the Tukey test ($p \leq 5\%$), using the Sisvar program.

Conclusions

The most appropriate procedure for conducting the tetrazolium test to evaluate the viability and vigor of *M. oleifera* seeds is the use of a concentration of 0.05% for 3 hours of immersion in salt.

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