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# Seed viability and vigour of two nanche species (*Malpighia mexicana* and *Byrsonima crassifolia*)

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## Abstract

This research aimed to determine the optimal pre-treatment, tetrazolium concentration and staining times to evaluate the biological condition of the embryos in two nanche species, *Malpighia mexicana*, collected in Matatlan, and *Byrsonima crassifolia*, collected from Tehuantepec, Oaxaca. The fruit pulp was removed and the seeds were washed and dried. The embryos were placed in 0.1 or 1% tetrazolium for 18, 24 or 48 hours at 30°C. A random design was used with 12 treatments consisting of four replications of 25 embryos each. Viability and vigour were evaluated based on embryo colour patterns. *M. mexicana* seeds showed 90% viable vigorous embryos when pre-treated for 24 hours and stained with 0.1% tetrazolium for 48 hours, whereas *B. crassifolia* seeds showed 90% with 1% tetrazolium for 48 hours. The tetrazolium test allowed for assessment of embryo vigour by the pattern differences and colour intensity obtained.

## Introduction

Seeds are essential for the multiplication of plant species (Victoria *et al.*, 2006) and their quality is crucial for obtaining good harvests (Ávila-Marioni *et al.*, 2012). The International Seed Testing Association investigates and publishes procedures for the analysis of seed quality, primarily of economically profitable crops (Victoria *et al.*, 2006). However, there are species that require more research because they have important features for human wellbeing (Victoria *et al.*, 2006). Such is the case of species from the family Malpighiaceae, on which little research has been carried out in relation to the tetrazolium test for seed viability and vigour (Costa *et al.*, 2003; Jaimes *et al.*, 2014). The seeds of species belonging to this family, distributed in humid and dry tropical areas (Araújo and Minami, 1994) such as Brazil, Honduras, Mexico and Florida, USA (Anderson, 1979; García-Hoyos *et al.*, 2011), have low viability and germination (Gomes, 2001; Costa *et al.*, 2003).

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In Mexico, *Malpighia mexicana* A. Juss. is known as a red nanche, guajocote, nanche de monte, nance and nanche; *Byrsonima crassifolia* (L.) H.B.K. is known as yellow nanche, nance, changunga and nanche (Herrera-Ruiz *et al.*, 2011). The fruits are drupes. *M. mexicana* has a red peel and three fibrous endocarps, usually with one or two developed embryos (Costa *et al.*, 2003; Souto and Oliveira, 2008); in contrast, the yellow nanche has a yellow, orange and brown peel and a hard endocarp with three cavities (Maldonado *et al.*, 2014), usually with one or two developed embryos per cavity (Azeredo *et al.*, 1994; Nacif *et al.*, 1996). Both species were used by pre-Hispanic cultures as an alternative medicine (Ferreira *et al.*, 2005; Maldini *et al.*, 2009). Nanche fruits contain high levels of ascorbic acid, antioxidants and phenolic compounds, which are good for human health. The nanche fruits are consumed in Mexico as fresh fruits, frozen fruit, juice and liqueur, among others (Lima *et al.*, 2014; Mariutti *et al.*, 2014).

The tetrazolium (2,3,5-triphenyl tetrazolium chloride) test is one of the official methods for the evaluation of viability and vigour in seeds (Piña-Rodrihes *et al.*, 2004). It is based on biochemical reactions of certain enzymes in living cells (Moreno, 1984). These reactions occur within the cells and the red pigment formed (formazan) is insoluble, preventing diffusion of colour to other cells and allowing dead cells to retain their original white colour (França *et al.,* 1998). The activity of the enzyme systems decrease with the viability of the seeds; a deep red colour indicates the presence of living cells in the embryo, whereas pink, pale pink or the natural white colour, which is seen in the absence of an enzymatic reaction, indicate low viability or the death of the cells (Moreno, 1984; Russi *et al.*, 2010). This test also shows damages caused by insects or fractures or a lack of embryo maturity (Ruiz, 2009). To optimise the test, it is important to consider the concentration of tetrazolium, staining period and temperature, which vary with the species and even between seeds of the same sample (Victoria *et al.*, 2006); however, staining is generally faster in concentrated solutions at high temperatures (Peretti, 1994; Mello and Tillmann, 2001).

Seed vigour is an indicator of the physiological quality of the seeds (Spina and Carvalho, 1986), and it is the sum of those properties that determine the level of activity and performance during germination and emergence of seedlings (ISTA, 2005). Vigorous seeds have a quick and uniform emergence with normal seedlings that can easily adapt to a wide range of environmental conditions (Carvalho and Nakagawa, 2000; Krzyzanowski and França Neto, 2001).

There has been some research on Malpighiaceae related to planting, asexual propagation and morphological and anatomical descriptions of their plant organs (Laskowski and Bautista, 1998, 2003; Mondin *et al.*, 2010; Barbosa *et al.*, 2014). However, research related to seed quality, germination, viability, dormancy and accelerated ageing is scarce. Thus, the aim of this research was to determine the optimum periods of pre-treatment and staining, and concentration of tetrazolium to evaluate the viability and vigour of the seeds of two species of nanche (*M. mexicana* and *B. crassifolia*).

## Materials and methods

*Malpighia mexicana* fruits were collected from wild bushes in the municipal area of Santiago Matatlan, Oaxaca. *Byrsonima crassifolia* fruits were obtained in the central market in Oaxaca City and were originally harvested from trees grown in the background orchards in Santo Domingo, Tehuantepec, Oaxaca, in July 2014. Matatlan is located at 16°51'39.28" N and 96°22'50.11" W at an altitude of 1,729 m a.s.l. and the weather in this region is warm and sub-humid with rain in the summer (INEGI, 2012). Tehuantepec is located at 16°19'28" N and 95°14'20" W at an altitude of 50 m a.s.l. and the weather is hot and tropical, with scarce thermal oscillation year round (INEGI, 2012). The fruits were taken to the Seed Analysis Laboratory of Colegio de Postgraduados, and only the healthiest, most complete and homogenously sized fruits were chosen. They were washed and later rubbed in a rectangular metal sieve (5 mm) until the greatest amount of pulp possible was removed; endocarps were rinsed and dried for 72 hours at room temperature (25°C). The endocarp base was then cut off on the opposite side of each embryo radicle (figure 1) in order to facilitate the entry of water, the separation of the testa and the elimination of the integument.

Samples of 25 complete embryos were placed into small, labelled containers containing 25 ml distilled water for 18 or 24 hours. After that, the embryos were submerged in 0.1 or 1% tetrazolium solutions for 18, 24 or 48 hours in the absence of light, and the containers were placed in an oven (Central Scientific Division of CENCO) at 30°C. This combination allowed for 24 treatments consisting of four replications of 25 embryos arranged in a random design. After each treatment, the embryos were removed from the tetrazolium solution, rinsed with distilled water and kept on moist towels during evaluation. The evaluations were carried out based on careful observations and by applying criteria from the staining patterns observed in the nanche seeds and defined standards for other species. The variables evaluated were the number of viable and not viable embryos, and the percentage of viability and vigour based on colour acquired. All embryos with an intense red colour were graded as vigorous, those with a slightly pale colour were graded as viable but with low vigour, and those with no colour were graded as dead embryos. The embryos subjected to viability and vigour tests with tetrazolium were schematically represented according to the staining category suggested by ISTA (2010) for species that produce seeds with a hard testa. Images of the embryos were obtained with a Carl Zeiss Tessovar microscope and a digital camera for microscopy (PAXcam 3). Then, the images were improved using GIMP software, version 2.8.14. The data were arcsine-transformed and analysed by ANOVA and Tukey's multiple comparisons test using the statistical package SAS® 9.2 (SAS Institute, 2009).

*Embryo evaluation*

Figure 2 shows the categories of tetrazolium staining: vigorous viable embryos, embryos with low vigour and non-viable embryos. For *M. mexicana*, figure 2a corresponds to an intense red coloured embryo without defects and considered viable and with high vigour. Figure 2b shows a red embryo with white areas in the cotyledons, which may indicate initial stages of loss of viability. However, it is considered a viable and vigorous

embryo due to the lack of damage in the radicular apex. The embryos shown in figure 2c and 2d display intense red colours in parts of the apices of the cotyledons, indicating viability and vigour, yet over one-third are pale pink in the radicle area, indicating low viability and vigour, which may lead to abnormal or non-emergent seedlings. Finally, the embryo in figure 2e is complete but with no staining, showing no viability and therefore no vigour.

*Malpighia mexicana* A. Juss. *Byrsonima crassifolia* (L.) H. B. K.

Figure 1. Transversal cut of endocarps made at the opposite side of the embryo radicle to accelerate the imbibition.

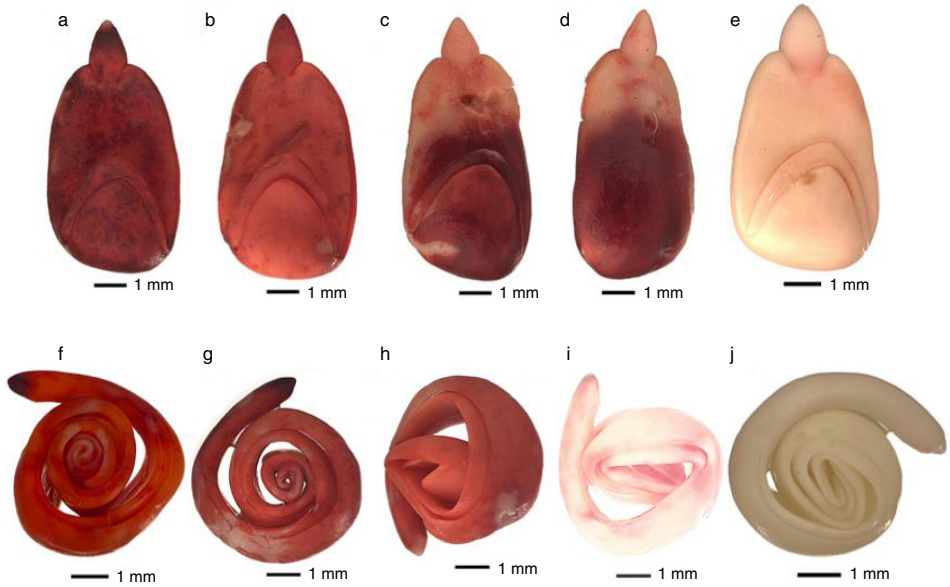


Figure 2. Embryos of two species of nanche, *Malpighia mexicana* A. Juss. (a-e) and *Byrsonima crassifolia* (L.) H. B. K., stained with tetrazolium. a, b, f-h = viable and vigorous embryos; c, d, i = viable embryo with low vigour; e, j = dead embryos.

In the case of *B. crassifolia*, figure 2f indicates embryos with good viability and high vigour; the red colour is intense and it is easy to see activity in the radicular apex, so seedlings will have potential for good growth and establishment in the field. The embryo in figure 2g is red with small white spots in the area of the cotyledons but an intensely coloured radicle, indicating viability and vigour. The embryo in figure 2h is light red and shows damage in the cotyledons and less colour in the radicular apex, indicating a reduction in viability and vigour. The embryo in figure 2i is completely white with small pink areas, indicating advanced cell death and consequently, low viability and vigour, such that it will be unable to produce a vigorous seedling. Finally, the embryo in figure 2j has well-defined morphological characteristics but shows no respiratory cell activity or staining and is therefore considered a dead embryo.

## Results

In both *M. mexicana* and *B. crassifolia*, the results of viability and vigour tests were similar. The viability and vigour of the embryos in both nanche species as determined using 1% tetrazolium solution yielded 74.8% viable vigorous embryos in *M. mexicana* and 72.4% in *B. crassifolia* (table 1). The embryo staining was improved as the

Table 1. Percentages of viable vigorous embryos and low-vigour embryos of two nanche species depending on the test conditions (concentration of tetrazolium solution, length of imbibition period and length of time immersed in tetrazoilum solution).

| Concentration of TZ (%) | Imbibition period (hours) | Staining (hours) | Embryos | | | |
|---|---|---|---|---|---|---|
| | | | Viable and vigorous (%) | | Viable and low vigour (%) | |
| | | | *M. mexicana* | *B. crassifolia* | *M. mexicana* | *B. crassifolia* |
| 0.1 | 18 | 18 | 0 c | 0 e | 90 a | 90 a |
| 0.1 | 18 | 24 | 78.3 ab | 0 e | 11.8 bc | 90 a |
| 0.1 | 18 | 48 | 0 c | 23.5 de | 90 a | 66.7 ab |
| 0.1 | 24 | 18 | 78.3 ab | 0 e | 11.8 bc | 90 a |
| 0.1 | 24 | 24 | 47.4 b | 47.4 cd | 42.8 b | 40.4 bcd |
| 0.1 | 24 | 48 | 90 a | 33.3 cd | 0 c | 56.8 bc |
| 1 | 18 | 18 | 0 c | 79.9 ab | 90 a | 10.2 ef |
| 1 | 18 | 24 | 54.9 ab | 76.8 ab | 35.3 bc | 11.8 ef |
| 1 | 18 | 48 | 49.7 b | 76.1 ab | 40.4 b | 14.1 def |
| 1 | 24 | 18 | 56.9 ab | 58.2 bc | 33.3 bc | 31.9 cde |
| 1 | 24 | 24 | 56.9 ab | 54.4 bc | 33.2 bc | 35.7 cde |
| 1 | 24 | 48 | 90 a | 90 a | 0 c | 0 f |
| | | *CV* | 18.6 | 15.8 | 23.4 | 14.9 |
| *n = 1200 embryos* | | *HSD* | 37.1 | 28.2 | 37.1 | 26.6 |

Results with the same letters within a column are not significantly different (Tukey, $\alpha = 0.05$); TZ = tetrazolium; n = number; CV = coefficient of variation coefficient; HSD = honestly significant difference.

concentration of tetrazolium increased; a concentration of 0.1% indicated 64.6% viability in *M. mexicana* and 70.8% in *B. crassifolia*, but with low vigour because the colour was pale pink even though the entirety of the tissue was coloured. The embryos were pre-treated in distilled water, which is critical for the imbibition before staining. There were no significant differences in the observed viability depending on the period of imbibition. A greater effect of the amount of time in the tetrazolium solution was observed; 69.9% of *M. mexicana* embryos had an intense and uniform red colour after 48 hours in tetrazolium, indicating good viability and vigour, surpassing *B. crassifolia* (58.8%). Also notable were the percentages of viable embryos with low vigour that were obtained with 18 hours of staining; *M. mexicana* had 56.8% uniform pale pink embryos and *B. crassifolia* 58.3%, indicating that these species were capable of producing normal seedlings but possibly with slow germination or emergence problems due to low vigour.

In endocarps from *M. mexicana*, there were no dead embryos; *B. crassifolia* had low percentages of dead embryos, with no statistically significant differences between them. Concentrations of 1 and 0.1% of the tetrazolium solution resulted in 0.5 and 0.7% dead embryos, respectively; a similar result was obtained with pre-treatment in distilled water for 18 and 24 hours. Only 1.8% of the embryos were found uncoloured when staining lasted 24 hours.

In *M. mexicana*, the duration of pre-treatment in water and the staining time affected the test similarly because the use of 0.1 and 1% tetrazolium for 18 hours of imbibition and staining yielded viable embryos with little vigour. A high percentage of viable embryos with little vigour were also found in this species when using 0.1% tetrazolium for 18 hours imbibition and 48 hours staining. *B. crassifolia* showed high percentages of viable embryos with little vigour when using 0.1% tetrazolium for 18 hours imbibition and 18 hours staining, as well as 18 hours imbibition and 24 hours staining.

Additionally, in *B. crassifolia*, when using 0.1% tetrazolium for 24 hours imbibition and 24 hours staining, there were 8.3% new dead embryos versus 5.8% when using 1% tetrazolium for 18 hours of imbibition and 24 hours of staining.

## Discussion

Costa *et al.* (2003) evaluated *Malpighia emarginata* DC. embryos and found that using 0.5% tetrazolium for 12 hours staining yields an intense and uniform red colour, indicating viable and vigorous embryos. These results agree with those found in this investigation, in which embryos of both species behaved similarly in terms of viability and vigour.

Embryos that are uniformly pale pink are considered to have the capacity to produce normal seedlings, but possibly with slow germination or emergence problems due to their low vigour (Jorge *et al.*, 2006). This situation may be due to the low respiratory rate of embryo cells that could not reduce the tetrazolium to formazan or because the tetrazolium concentration was too low and did not react to form formazan. Salinas *et al.* (2001) mentioned that the seeds that had high viability and vigour were those that had the greatest possibilities of emerging and establishing without problems. However, the Malpighiaceae presented problems that may be due to some type of dormancy or

malformation of the ovule (Araújo and Minami, 1994). Jaimes *et al.* (2014) used embryos of seeds that had been stored for six months at -20°C and had variable moisture contents, and these embryos displayed viability with reduced vigour. Conversely, Martinelli (1997) and ISTA (2007) mention that in fodder species, an embryo is considered viable when it becomes coloured on over one-third of the radicle.

Germination in wild species is generally low, probably due to the seeds showing some type of dormancy, thus avoiding immediate germination (Gómes, 2001). Benito-Matias *et al.* (2004) note that the viability test with tetrazolium does not always predict the potential for germination of the seeds because every species is different and because seed quality varies depending on internal and external factors. In the tissues of embryos conditioned in water, respiration accelerates, and cell staining improves (Benito-Matías *et al.*, 2004); this corresponds with this investigation, which found that a greater time of imbibition correlated with better viability and vigour.

Studies carried out on acerola (*Malpighia punicifolia* L.) concluded that imbibition influences the viability and vigour of seeds (Azerêdo *et al.*, 2005). These results are consistent with those reported by Jaimes *et al.* (2014), who mentioned that embryos with scarce staining display little vigour for the production of a strong seedling (Moreno, 1996). However, this may be due to factors within or outside the seed itself (Salinas *et al.*, 2001).

## Conclusions

The embryos of *M. mexicana* and *B. crassifolia* require pre-treatment in distilled water for 24 hours and staining times of 48 hours. However, for greater efficiency in determining viability and vigour, *M. mexicana* requires a tetrazolium concentration of 0.1%, and *B. crassifolia* requires 1%.

The test with tetrazolium helped find different quality seeds and firmness levels, based on the colour pattern showed, in which an intense red indicated seeds with high viability and vigour, pale pink colours indicated low viability and vigour, and no colour indicated death of the embryo.

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