Topographic tetrazolium test in seeds of *Tillandsia ionantha* Planch.

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ABSTRACT

Objective: To develop a protocol proposal for the application of the tetrazolium test on *Tillandsia ionantha* Planch. seeds.

Design/methodology/approach: A factorial design was used, with the first factor corresponding to tetrazolium concentrations (0.1%, 0.5%, and 1%), the application or omission of preconditioning by soaking for 12 hours, and the presence or absence of physical scarification. This resulted in twelve treatments, each with three replicates of ten seeds per experimental unit. The seed coats were removed, and according to each treatment, seeds were soaked in sterile distilled water for 12 hours and subjected to physical scarification using water sandpaper. The seeds were then placed in plastic containers with lids at room temperature (23 °C) for 48 hours to assess staining percentage and topographic seed staining patterns.

Results: ANOVA (α=0.05) revealed that the only main factor capable of producing a statistically significant difference ($p \leq 0.05$) in seed staining —and therefore in viability detection— was seed preconditioning through 12-hour soaking. In contrast, neither scarification nor the tetrazolium concentrations tested had a significant effect on staining outcomes ($p > 0.05$).

Limitations on study/implications: The seeds of this species are very small, which makes them difficult to handle, and the seed coat sometimes prevents proper observation of the staining.

Findings/conclusions: To perform the test, the process will begin by removing the coma, followed by soaking the seeds for 12 hours.

Keywords: Bromeliad, germination, viability, protocol.

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INTRODUCTION

To develop *ex situ* seed conservation programs aimed at mitigating the effects of illegal exploitation, it is necessary to understand the germination characteristics of wild plant species (Flores *et al.*, 2015). Germination is defined as the process that begins with seed imbibition —that is, the absorption of water— and ends with the emergence of the embryonic axis through the surrounding structures (Bewley *et al.*, 2012).

To determine whether a seed sample has the capacity to germinate, seed viability tests can be conducted. According to Schmidt (2007), these include techniques for visually

evaluating seeds and assessing at least some critical physiological processes. Among these techniques is the tetrazolium test, which is based on the topographic evaluation of a biochemical assay that identifies living embryonic structures —those responsible for enabling seed germination (Dadlani & Yadava, 2023).

The principle underlying the test is that dehydrogenases are a class of metabolic enzymes present in cells with active metabolism, which release hydrogen during reduction processes. This hydrogen has the ability to convert a pale yellow solution —containing either 2,3,5-triphenyltetrazolium chloride or 2,3,5-triphenyltetrazolium bromide— into a stable red-colored compound known as triphenyl formazan. Consequently, the production of red formazan serves as an indicator of dehydrogenase activity, which in turn reflects metabolic activity and, therefore, cellular viability.

Because tissue staining is localized, it is possible to distinguish between the living (red-stained) and dead (unstained) sections of the seed. In cases where necrotic (dead) tissue is present only on the surface of the cotyledons while the radicle stains normally, the seeds may still retain their viability. In contrast, even small areas of necrotic tissue in the essential region of the embryo generally indicate that the seed is unlikely to germinate (Schmidt, 2007).

Some of the advantages of this test, as noted by Dadlani & Yadava (2023), are that it can be considered an alternative to standard germination tests, and it helps assess both the physical and physiological condition of seeds by detecting structural alterations and damage caused by insects or post-harvest handling. Additionally, the test is simple to perform, does not require expensive equipment, and is not influenced by environmental factors. However, to obtain more accurate results in tetrazolium testing for a specific seed species, Mancipe *et al.* (2018) indicate that it may be necessary to adjust the test conditions. To ensure the success of the test, factors such as tetrazolium concentration, staining duration, temperature, and correct interpretation of the staining pattern must be considered. Therefore, the objective of this study was to propose a protocol for the application of the tetrazolium topographic viability test on Tillandsia ionantha seeds, including seed pretreatments such as soaking, physical scarification, and the use of different tetrazolium concentrations.

MATERIALS AND METHODS

The topographic evaluation using the tetrazolium test on *Tillandsia ionantha* seeds was carried out at the Plant Tissue Culture Laboratory of the Departamento de Fitotecnia at the Universidad Autónoma Chapingo. Seeds were provided by the University Center for Research and Conservation of Mexican Bromeliads (CUCIBROM, by its initials in Spanish) and were stored at an average room temperature of 20 °C in plastic containers with lids.

To facilitate seed handling, the plumose appendage (coma) of each seed was removed using fine scissors and tweezers. Twelve treatments were applied, half of which included soaking the seeds in sterile distilled water for 12 hours. Additionally, half of the seed samples underwent physical scarification using water sandpaper. Finally, ten seeds were placed in each plastic container with a lid, to which tetrazolium solution was added at concentrations of 0.1%, 0.5%, and 1%. The treatments described above are summarized in Table 1.

Table 1. Treatments applied to *Tillandsia ionantha* Planch. seeds established in the tetrazolium test assay.

Treatment	Soaking (12 hours)	Physical scarification	Tetrazolium
T1	Yes	Yes	0.1
T2	Yes	Yes	0.5
T3	Yes	Yes	1
T4	Yes	No	0.1
T5	Yes	No	0.5
T6	Yes	No	1
T7	No	Yes	0.1
T8	No	Yes	0.5
T9	No	Yes	1
T10	No	No	0.1
T11	No	No	0.5
T12	No	No	1

The variables evaluated were, on one hand, the percentage of seeds showing staining in the embryonic area and radicle, as well as the staining pattern present in the seeds. These variables were assessed 24 hours after the establishment of the bioassay. Each treatment had three replications with $n=10$ seeds per replication, and the experimental design corresponded to a factorial design. Statistical analysis was performed using Minitab® 18.1 software.

RESULTS AND DISCUSSION

The percentage of seeds showing staining in the embryo and radicle areas was evaluated. Analysis of variance ($\alpha \leq 0.05$) showed that only the soaking factor (12 h) had a statistically significant effect on seed staining ($p \leq 0.006$). The interactions Tetrazolium-Physical Scarification, Tetrazolium-Soaking, and Physical Scarification*Soaking did not show statistically significant effects ($p \geq 0.05$). Finally, the interaction of all three factors evaluated was not statistically significant ($p \geq 0.293$) for seed staining (Table 2).

The Pareto chart of standardized effects ($\alpha = 0.05$) (Figure 1) confirms the results of the analysis of variance (ANOVA), showing that the only critical factor affecting staining is the seed soaking treatment, as it exceeds the critical value (2.064).

The Main Effects Plot for seed staining (Figure 2) shows that there is a greater effect or response on staining when seeds undergo a 12-hour pre-soaking before applying tetrazolium. However, as mentioned in the ANOVA, different tetrazolium concentrations and the application or omission of physical scarification do not have a significant effect on staining. In the Main Effects Plot, it can be observed that the 0.1% tetrazolium concentration has a greater effect than the 0.5% and 1% concentrations. Additionally, it can be seen that not performing physical scarification has a greater effect on potential seed staining.

Table 2. Analysis of variance of the factors tetrazolium (TZ), physical scarification (PS), and soaking (SO). DF=degrees of freedom, SS=sum of squares, MS=mean squares.

Source	DF	SS	MS	Value F	Value p
Model	11	0.004244	0.000386	1.84	0.102
Lineal	4	0.002052	0.000513	2.45	0.073
TZ	2	0.000123	0.000061	0.29	0.748
PS	1	0.000063	0.000063	0.30	0.588
SO	1	0.001866	0.001866	8.92	0.006
Interaction 2 terms	5	0.001650	0.000330	1.58	0.205
TZ*PS	2	0.000108	0.000054	0.26	0.775
TZ*so	2	0.001086	0.000543	2.60	0.095
PS*SO	1	0.000456	0.000456	2.18	0.153
Interaction 3 terms	2	0.000541	0.000271	1.29	0.293
TZ*PS*SO	2	0.000541	0.000271	1.29	0.293
Error	24	0.005022	0.000209		
Total	35	0.009266			

Statistically significant difference ($p \leq 0.05$).

Staining Topography

Regarding the staining topography of the seed due to the effect of tetrazolium, it was determined that the stained parts correspond to the cotyledon, hypocotyl, and root (Figure 3). Although physical scarification does not affect tetrazolium staining, it proves to be a useful technique to facilitate the observation of the internal staining pattern, since the seeds are very small and covered by the coma, making it difficult to see the staining pattern.

Staining pattern scale

Seven staining patterns were identified in the seeds, ranging from seeds with a complete absence of staining, staining in the root area, staining in the root and hypocotyl, staining in

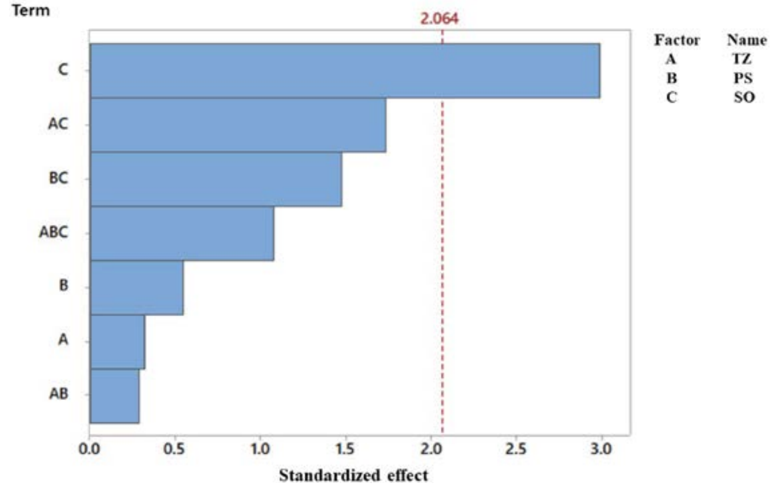

Figure 1. Pareto chart showing that only the seed soaking factor is a main effect. (Critical value=2.064, TZ=Tetrazolium, PS=Physical Scarification, SO=Soaking)

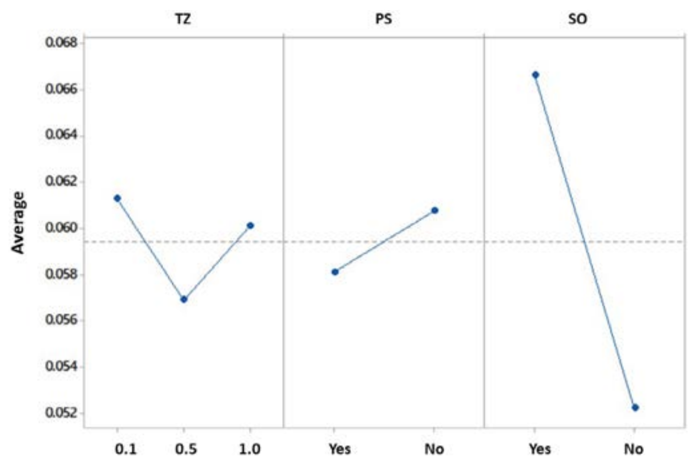

Figure 2. Main effects plot of seed staining in *Tillandsia ionantha* Planch. for the factors tetrazolium dose, physical scarification, and soaking. TZ=Tetrazolium, PS=Physical Scarification, SO=Soaking.

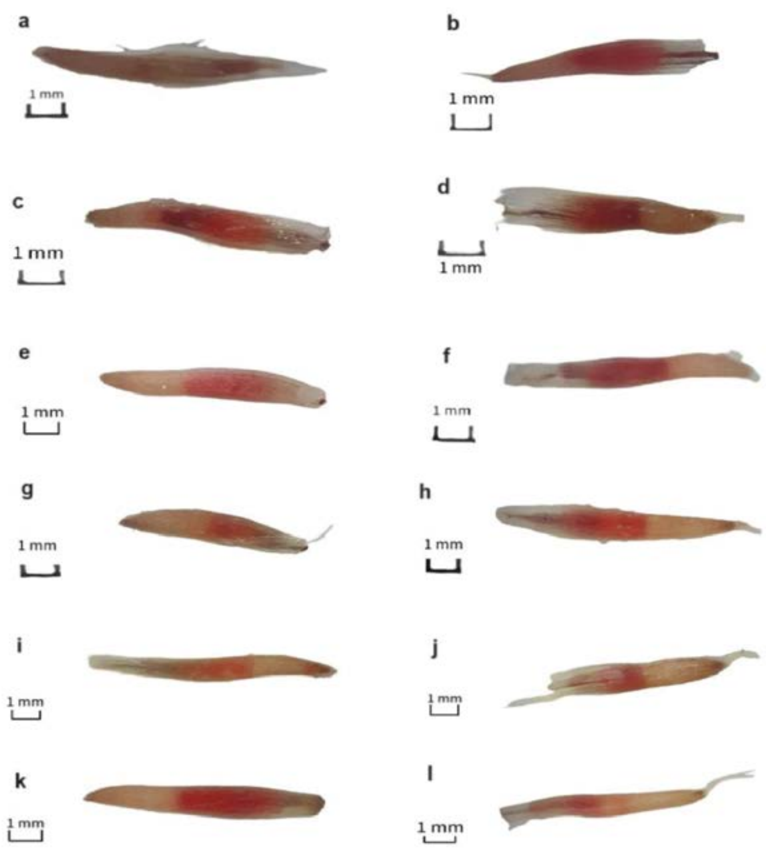

Figure 3. Staining pattern of *Tillandsia ionantha* Planch. seeds under different treatments. a) T1: Tetrazolium 0.1 N, with physical scarification and soaking; b) T2: Tetrazolium 0.5 N, with physical scarification and soaking; c) Tetrazolium 1 N, with physical scarification and soaking; d) Tetrazolium 0.1 N, without physical scarification and with soaking; e) Tetrazolium 0.5 N, without physical scarification and with soaking; f) Tetrazolium 1 N, without physical scarification and with soaking; g) Tetrazolium 0.1 N, with physical scarification and without soaking; h) Tetrazolium 0.5 N, with physical scarification and without soaking; i) Tetrazolium 1 N, with physical scarification and without soaking; j) Tetrazolium 0.1 N, without physical scarification and without soaking; k) Tetrazolium 0.5 N, without physical scarification and without soaking; l) Tetrazolium 1 N, without physical scarification and without soaking.

the cotyledon and hypocotyl, staining in the cotyledon and root, seeds with staining only at the cotyledon level, to seeds showing total staining of the embryonic area, which includes the root, hypocotyl, and cotyledon. The staining topography caused by tetrazolium indicates that seeds are viable when the staining in the embryonic area is darker. Likewise, when there is no staining or it is barely perceptible with pinkish hues and the staining is not uniform, the seed can be considered non-viable (Figure 4).

Based on the results obtained and in contrast to what was reported by Calderón (2019), who indicated that in *Espeletia* and *P. ledifolia*, the most important factor for staining was the concentration of tetrazolium —since when 1% tetrazolium was applied for 24 h, viability reached 76% and 80%, respectively— in the case of *T. ionantha* seeds, it was determined that the main factor affecting staining was the prior soaking of the seeds for 12 hours, rather than the tetrazolium concentration.

Regarding the anatomical structure of the seeds, Calderón (2019) indicates that in the cases of *H. goudotiana*, *C. buxifolium*, and *X. spiculifera*, which are characterized by having either a thick seed coat or abundant endosperm, the test must be applied directly to the embryos using tetrazolium at a 1% concentration. Additionally, the author mentions that for *G. anastomosans*, *P. prostrata*, and *V. floribundum*, which have a hard seed coat, it is necessary to cut the seed coat and increase the tetrazolium concentration to 1.5% in order to obtain viability values of up to 40%. However, *T. ionantha* seeds do not exhibit these anatomical characteristics, which facilitates the direct application of the tetrazolium test without the need to cut or physically scarify the seed coat to allow the solution to permeate the seed tissue.

In the study conducted by Elizalde (2014) with *H. perotensis*, it was determined that, similar to the seeds of *T. ionantha*, tetrazolium concentrations of 0.2% and 1% did not show statistically significant differences ($p>0.05$) in embryo staining, allowing for the identification and assessment of seed viability. In viability tests using tetrazolium on

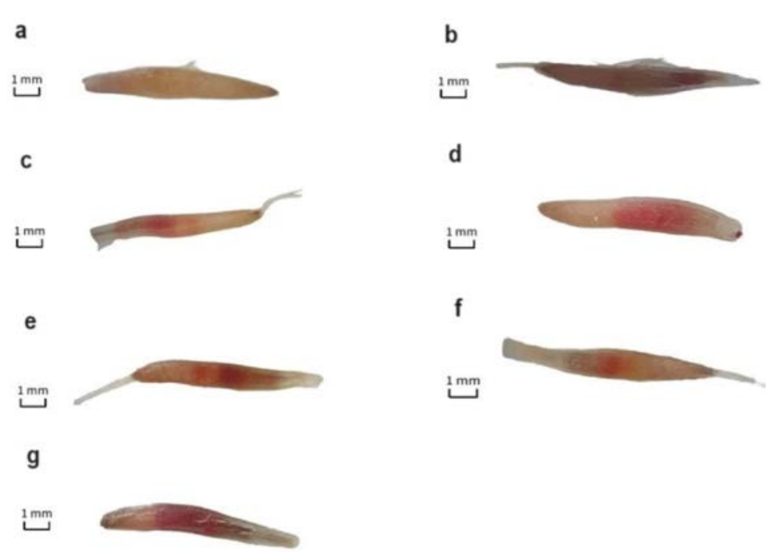

Figure 4. Staining patterns obtained from the different treatments, indicating the variety of areas within the seed that are capable of being stained by tetrazolium in *Tillandsia ionantha* Planch. seeds.

Hordeum vulgare L., authors such as Grzybowski *et al*. (2012) determined the usefulness of pre-conditioning seeds by directly immersing them in water. Unlike *T. ionantha* seeds, in barley it is sufficient to apply pre-conditioning for only four hours. They also concluded that effective tetrazolium concentrations for staining barley seeds are 0.1% or 0.5%, which aligns with the results obtained for *T. ionantha*, where seed staining also occurred at those concentrations. Mollo (2011) reported that seeds of *Hordeum muticum* and *Bromus catharticus* did not show sensitivity to the evaluated concentrations (0.1%, 0.5%, and 1%), and viability was observed at any of these doses, which is consistent with the behavior observed in *T. ionantha*.

Finally, it is important to highlight, as noted by Elizalde *et al*. (2017), that the application of the tetrazolium test depends on the evaluator's experience in detecting seed viability. In addition, it is necessary to develop specific protocols to assess viability in bromeliad seeds, which are poorly documented —partly, according to the authors, due to the notably small size of the seeds in this family.

CONCLUSIONS

The proposed protocol for the tetrazolium viability test in *T. ionantha* seeds should begin with the removal of the coma and soaking the seeds in sterile distilled water for 12 hours. Tetrazolium should be applied at a concentration of 0.1%. Physical scarification may be optionally included as a preconditioning step, solely to facilitate the visualization of staining patterns due to the small size of the seeds.

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