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Pregerminative treatments in *Tillandsia ionantha* seeds to obtain seedlings under *in vitro* culture

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ABSTRACT

Objective: To search for an *in vitro* strategy to favor both germination and a greater number of seedlings in *Tillandsia ionantha*; also, to promote the development of future research on this species.

Design/methodology/approach: Factor one: lighting conditions (light-dark), factor two: 13 preconditioning treatments, which included storage at room temperature and in refrigeration at 10 °C, soaking (12 and 24 hours), with hydrogen peroxide (10 and 20%), potassium nitrate (0.2 and 0.4%), gibberellins (50 and 150 $ml.l^{-1}$), three alternate incubation temperatures (28, 32 and 36 °C). They were sown in MS medium (Murashige and Skoog, 1962) at 25%, adding 20 $g.l^{-1}$ of sugar, 2 $g.l^{-1}$ of activated carbon, and 5.5 $g.l^{-1}$ of agar. A flask with three seeds which coma removed was established as an experimental unit; 15 repetitions were established and placed in the incubation room at 24 °C with a photoperiod of 16:8. The germination process was recorded, and the seedlings were extracted two months after their establishment.

Results: The treatment that resulted in the highest number of seeds that initiated the germination process and the highest number of plants was when the seeds were kept at room temperature. The highest contamination was observed in the treatment exposed to 32 °C. It was observed that 80% of the experimental units showed signs of imbibition within a few days, although the vast majority did not complete the process and the maximum yield was on average 1.4 seedlings.

Conclusions: The best treatment is to use seeds stored at room temperature and if storage is necessary, to soak them for 12 h.

Keywords: native, conservation, bromeliad.

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INTRODUCTION

Illegal trade of exotic plants and other lifeforms is a common practice in the world. This article seeks to promote alternative forms of production and propagation of certain genera of Bromeliaceae, which occupy the eleventh place in specific wealth among angiosperms and the third place among monocotyledons in Mexico. The genus *Tillandsia* at the global level has more than 649 species and includes members with distinctive morphological and physiological

characteristics (Pickens *et al.*, 2006; Gouda *et al.*, 2016; Granados *et al.*, 2016). Most of the species of the genus are epiphytes or live on rocky hillsides, preferring to become established on tree branches of *Quercus* spp. due to its coarse bark. They have leaves covered with pelted trichomes and are arranged in rosettes, while the inflorescences are bracts with bright colors (Diego-Escobar *et al.*, 2013); in Mexico, *Tillandsia* occupies the first place with 230 species. *T. ionantha* is an example of these and is generally traded illegally; it is commonly known as an air plant with compact rosette growth that has red pigment in certain seasons. Therefore, an *in vitro* strategy is sought to favor both the germination and a larger number of seedlings in *Tillandsia ionantha*, in order to promote the development of future research on this species.

MATERIALS AND METHODS

Location of the study

The study was developed in the Laboratory of Plant Tissue Cultivation and seeds, of the Plant Production Department at Universidad Autónoma Chapingo; *T. ionantha* seeds were used from wild plants from the state of Morelos. The growth medium was Murashige and Skoog (1962) at 25%, with addition of 20 g L^{-1} sucrose and 2 g L^{-1} activated carbon, pH adjusted to 5.7±1; 5.5 g L^{-1} Deiman® brand agar as gelling agent was added to 10 mL of the medium in a Gerber® brand jar, which was then introduced into an autoclave to be sterilized for 25 min at a temperature of 121 °C and a pressure of 1.1 to 1.2 kg cm^{2-1}.

Preconditioning of seeds

The coma (crest of hairs that ease the dispersion through wind) was eliminated from the seeds to avoid possible sources of contamination. Groups of 100 seeds were quantified and placed in filter paper envelopes to facilitate their management in each treatment. Once the pre-treatment was done, the seeds were washed inside the leaf flow chamber with the following procedure: a solution of 10% of commercial chlorine was added for 10 minutes, and after this time they were washed three times; a solution of 10% alcohol at 96% was added for 10 min, and after this time they were washed three times with sterile water; then a solution of 1.5 mL L^{-1} colloidal silver was added for 10 min, and after this time they were washed five times with sterile water.

Once the seeds were washed, the envelopes were opened individually and three seeds were sown per jar, then they were sealed with film tape and labeled; then they were incubated in the predetermined conditions. Concerning the light factor, it was supplied with a fluorescent lamp with a designation of cold white color (CW) with color code 33 with a relative color temperature of 4100 K and a mean design lumen of 9000 lm with nominal chromatic reproduction index of 62 and nominal potency of 185 Watts and exposure to a photoperiod of 16 light hours and 8 dark hours.

Treatments

Twenty-six treatments were established, which consisted in ten preconditioning and three incubation conditions. The control consisted in seeds that were stored since the

day of harvest at room temperature in the laboratory, the rest of the seeds were stored in refrigeration at 10 °C for 15 days and a group was established with this characteristic; seeds with previous soaking of 12 to 24 h were established, the treatments were exposed to a solution described in Table 1, immersed during 24 h in constant agitation. Other groups of seeds were incubated at different temperatures. All of these treatments (Table 1) were incubated in two conditions, light and dark.

Experimental design and establishment

The experimental design was completely random factorial with 15 repetitions, where each experimental unit was a jar with three seeds.

Response variables

The experiment was monitored three times per week, recording the following variables. Germination: a seed was considered germinated when the imbibition process was visible. Percentage of germination: it was evaluated in function of the number of germinated seeds from the total deposited in each jar and according to the date of data collection. Percentage of necrotization: it was evaluated in function of the number of seeds that visibly presented this condition according to the total deposited in each jar. Percentage of contamination: the jar that presented signs of contamination, whether fungus or bacteria, was eliminated and the seeds in them were counted as contaminated; in addition, in some jars the proliferation only affected one or two specimens, and they were the only ones quantified. Percentage of normal plants: at the end of the experiment the plants that did not present any type of physical abnormal condition, or any damage from contamination or necrotization, were extracted and quantified according to the treatment that they belonged to.

Table 1. Pregerminative treatments evaluated in *Tillandsia ionantha* Planch seeds.

Pregerminative	Treatments	
	Incubation condition	
	Light	Darkness
Room temperature	T1	T1
Cold stored	T2	T2
Soaking 12 hrs	T3	T3
Soaking 24 hrs	T4	T4
Hydrogen peroxide 10 %	T5	T5
Hydrogen peroxide 20 %	T6	T6
Potassium nitrate 0.2 %	T7	T7
Potassium nitrate 0.4 %	T8	T8
Gibberellins 50 mg.l	T9	T9
Gibberellins 150 mg.l	T10	T10
Incubated 28 °C	T11	T11
Incubated 32 °C	T12	T12
Incubated 36 °C	T13	T13

Statistical analysis

To analyze the variables, analysis of variance was used and since there were differences, Tukey's means test (p=0.05) was applied using the statistical software SAS version 9.0.

RESULTS AND DISCUSSION

Light factor

Regarding factor 1, no significant differences were found except at two days after sowing when the germination was higher with the light condition with 39.974%, the dark condition with 31.559% which was provided by keeping them in totally black boxes on both sides and an exposure of 24 h darkness; this could be contradicted by what was observed by Vadillo *et al.* (2004) when they evaluated *Puya raimondii* Harms seeds when considering the positive photoblast seeds, since *Tillandsia ionantha* germinated similarly in both conditions. However, since differences were found in terms of contamination, these could be attributed to the microclimate that was generated in the germination chamber, since in order to establish darkness cardboard boxes were designed within these pieces of equipment and with the moisture fungal formation colonies were observed around the box, which could inoculate the culture mediums within that box.

Beginning of germination

According to Tukey's test (p=0.05) at two days after sowing (das), the treatments T1, T3, T6 and T7 are statistically equal; however, it should be mentioned that treatment T1 (room temperature) was the one that presented a higher mean (64.567%) with specimens in phase 1 (Figure 1), which is why we could assume that germination of *Tillandsia ionantha* seeds is promoted under storage conditions at room temperature, as indicated by Klekailo *et al.* (2012), where the authors point out that temperature was a key factor, because germination was only found (a reduced fraction of the seeds and slowly) in the treatment at 20/30 °C; on the other hand, treatments T4, T5, T7, T9, T10, T11 and T13 are statistically equal, with treatment T9 (gibberellins 50 $mg.l^{-1}$) being the one that presented the lowest mean (13.2%) with specimens in phase 1, which is why we can speculate that the use of this plant hormone at this dose does not promote germination in this species. In this regard, Saldívar Iglesias *et al.* (2010) indicate that gibberellic acid in *Jaltomata procumbens* at a dose of 250 $mg.l^{-1}$, increased the speed compared to that reported in treatments of 200 and 150 $mg.l^{-1}$ and that the slower germination speeds are found with treatments of 100, 50 and 0 $mg.l^{-1}$ gibberellic acid; in general it was observed that as the concentration of gibberellic

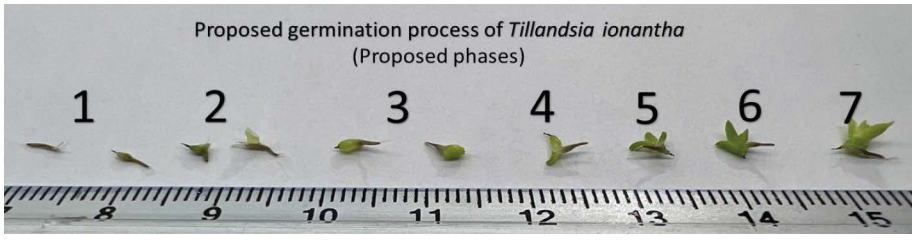

Figure 1. Germination process of *Tillandsia ionantha* Planch.

acid increases, the germination speed increases. According to the means test, at 4 (das) the treatments T1, T3, T6 and T8 are statistically equal; however, it should be mentioned that again the treatment T1 (room temperature) was the one that presented a higher mean (83.433%) with specimens in phase 1 (Figure 1). On the other hand, treatments T2, T4, T5, T7, T9, T10, T11 and T13 are statistically equal; however, it should be mentioned that the treatment T10 (gibberellins 150 $mg.l^{-1}$) presented the lowest mean (30.967) with specimens in phase 1 (Figure 1), which is why we can speculate that it contradicts what was observed by Saldívar Iglesias *et al.* (2010), since it indicates that with a higher concentration of gibberellins a higher germination speed was not observed in comparison to other treatments.

According to Tukey's test (p=0.05) at 7 (das) the treatments T1, T3, T6 and T8 are statistically equal; however, it should be mentioned again that the treatment T1 (room temperature) was the one that presented a higher mean (83.433%) with specimens in phase 1 (Figure 1). On the other hand, treatments T2, T4, T5, T7, T9, T10, T11 and T13 are statistically equal, although it should be mentioned that treatment T11 (incubated at 28 °C) presented the lowest mean (48.833%) with specimens in phase 1 (Figure 1).

Germination

According to Tukey's test (p=0.05), the treatments T1, T3, T5,T6, T7, T8, T11, T12 and T13 are statistically equal, although it should be mentioned that treatment T1 (room temperature) was the one that presented a higher mean (95%) of germinated specimens, and until this moment it could be seen that in order to guarantee a higher percentage of germination, it is not necessary to conduct pre-conditioning; regrettably, due to logistics the seed lots must be stored, generally under refrigeration to maintain the quality and health, according to what is described by Márquez (2019) who point out that seeds can remain viable for 38 months under refrigeration conditions at 10 °C with average germination of 78.9%, while, this study shows 73.467% under this condition; however, some pregerminative treatments promoted an increase in germination, almost equaling the seed without refrigeration.

Table 2 shows that the lowest percentage of germination is with seeds stored in cold (treatment T2) as well as continuous soaking for 24 hours (treatment T4). The latter is possibly linked to the seed's biology, and no information was found regarding the maximum imbibition of seeds of this species or similar, which is why it could be speculated that they suffered drowning.

Necrotization

Necrotization was present in all the treatments, with treatment T1 being the lowest with 48.867% which could agree with what was exposed by Calderón *et al.* (2011) when they pointed out that due to the biology of the wild Tillandsias, they can be very sensitive to the concentration of salts since a higher percentage of necrotization was actually observed in the seeds subjected to treatments such a potassium nitrate, hydrogen peroxide, and gibberellins, although 25% of the DM was used. Necrotization was possibly promoted

Table 2. Responses observed in the 26 pregerminative treatments in *Tillandsia ionantha* Planch.

Treatments	2 Days after sowing	4 Days after sowing	7 Days after sowing	% Germination	% Necrosed	% Contamination	% Normal plants
Light	39.97^{a}	49.35^{a}	60.87^{a}	87.80^{a}	75.27^{a}	5.9	19.61^{a}
Darkness	31.55^{b}	50.42^{a}	64.56^{a}	85.06^{a}	70.50^{a}	10.9	16.37^{a}
T1	64.56^{a}	83.43^{a}	91.17^{a}	95.6^{a}	48.87^{c}	6.67^{b}	46.7^{a}
T2	41.06^{bcd}	48.9^{bcd}	54.53^{ef}	73.47^{d}	73.4^{abc}	7.77^{b}	13.27^{bc}
T3	55.5^{ab}	65.6^{ab}	74.53^{abcd}	92.3^{abc}	62.37^{bc}	4.43^{b}	31.0^{ab}
T4	32.1^{cdef}	42.23^{cd}	54.53^{ef}	73.47^{d}	65.77^{abc}	6.67	13.2^{bc}
T5	25.36^{def}	36.5^{cd}	48.9^{f}	81.3^{abcd}	84.467^{ab}	0^{b}	10^{bc}
T6	54.53^{ab}	75.66^{a}	82.3^{ab}	94.5^{ab}	81.2^{ab}	11.1^{b}	14.3^{bc}
T7	27.67^{cdef}	41.03^{cd}	60.17^{cdef}	91.2^{abc}	89^{a}	0^{b}	9.9^{bc}
T8	46.73^{abc}	64.53^{ab}	77.93^{abc}	95.6^{a}	68.9^{abc}	16.6^{ab}	24.4^{bc}
T9	13.2^{f}	33.17^{cd}	48.87^{f}	79.03^{bcd}	88.93^{a}	5.53^{b}	5.533^{c}
T10	19.87^{ef}	30.97^{d}	48.9^{f}	76.83^{cd}	87.83^{a}	7.73^{b}	3.333^{c}
T11	17.7^{f}	32.07^{d}	48.83^{f}	90.1^{abc}	73.4^{abc}	12.17^{ab}	18.83^{bc}
T12	38.93^{bcde}	52.27^{bc}	69.07^{bcde}	90.1^{abc}	48.93^{c}	30^{a}	22.167^{bc}
T13	27.73^{cdef}	42.2^{cd}	55.6^{def}	90.1^{abc}	74.43^{ab}	1.1^{b}	21.167^{bc}

Averages per column with different letters indicate a significant difference according to Tukey's test (p=0.05).

when maintaining the seedlings during 60 days in this medium.

Contamination

According to Tukey's test (p=0.05), the treatments T11 and T12 are statistically equal, although it should be mentioned that treatment T12 (incubated at 32 °C), was the one that presented a higher mean (30%) with specimens contaminated as described previously; the highest contamination was seen in the germination chambers in which these treatments were found, as consequence of the microclimate generated inside them. The treatments T5 (hydrogen peroxide 10%) and T7 (potassium nitrate 0.2%) presented the lowest percentages of contamination with 0% both.

Normal plants

Although in percentages higher than 70% of germination were found all the treatments (Table 2), at 60 days when the seeds were extracted and a loss of more than 50% of specimens was found, due to contamination, oxidation or a case that was not reported where the seeds presented some of the germination phases, primarily phase 1 or 2 (Figure 1), and remained there without generating complete or normal seedlings.

CONCLUSION

The disinfection technique used was adequate, as well as the culture medium, although

it is recommended to conduct trials with low concentration of salts or monthly or biweekly replacements of the explants to decrease the oxidation percentages. Likewise, using seeds stored at room temperature eliminating the coma. The best treatment is to use seeds stored at room temperature and if their storage is necessary, to use soaking for 12 hours and tentatively potassium nitrate at a concentration of 0.4%, addressing the replacements or the decrease in salts to decrease possible causes of oxidation.

Differences were not seen between the treatments of light and dark.

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