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Factors affecting bud induction in *Fitzroya Cupressoides* (alerce) embryos cultured *in vitro*

Mutio Y.C.[1,2,3], Gallo A.G.[1,2,4], Moncaleán P.[5], Vélez M.L.[1,2,4*]

[1]Centro de Investigación y Extensión Forestal Andino Patagónico (CIEFAP)-Argentina, [2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)- Argentina, [3]Secretaría de Ciencia y Tecnología del Chubut- Argentina, [4]Universidad Nacional de la Patagonia San Juan Bosco- Argentina, [5]NEIKER-BRTA-España

*mvelez@ciefap.org.ar

INTRODUCTION & AIM

***Fitzroya Cupressoides* or 'alerce'**

'Alerce' is an endangered conifer native to Patagonia. The conservation of this species has gained increasing importance due to its significant potential for dendrochronological studies, as it ranks as the second longest-living species in the world..

Seed production is cyclic; seeds are frequently inviable in a high proportion, present dormancy, and there are no studies of their viability under long-term storage conditions.

These difficulties impact their use in *ex situ* conservation programs. Thus, *in vitro* culture may provide valuable tools for the propagation of alerce.

Our aim was to evaluate the influence of stratification, scarification, and sterilization procedures; explant type; basal culture media composition; and 6-benzyladenine (BA) cytokinin concentration on the success of bud induction.

MATERIALS AND METHODS

Plant material. Mature seeds were collected from stands located in the 'Los Alerces' National Park (71°53'47.09"W, 42°40'22.71"S) and in the 'El Turbio' Provincial Reserve (71°47'56.42"W, 42°17'48.70"S) in April 2023 and in April 2024. Seeds were stored clean and dry at 4-5 °C in sealed containers.

Experiment 1

-Set 1 treatments. Seeds were rinsed under running water for 5 min, followed by 1) short disinfection in 3% H_2O_2, 2) four different periods of stratification at 4°C, 3) three sterilization methods, and 4) cultivation in Petri dishes (90x15 mm) containing 20 mL of each of the bud induction media: four basal media (LP[1,2], SH[3], and the same media but with half macronutrients: HLP, HSH) supplemented with 4.4, 22, or 44 µM BA. All media were also supplemented with 3% sucrose and 1% bacteriological agar. The pH was adjusted to 5.8 before autoclaving (121 °C, 20 min). Treatments were performed in triplicate with ten seeds per Petri dish. All cultures were laid on the growth chamber at a temperature of 21 °C, at a 16 h photoperiod with 120 mol $m^{-2}s^{-1}$ light intensity provided by full spectrum tubes.

Stratification. To break dormancy before organogenesis, the seeds underwent cold-moist stratification. Seeds were hydrated for 48 h, then drained, and placed in 30 µm thick polyethylene bags. They were stored at 4°C for 7,14, 30 or 60 days.

Sterilization. i) 3% H_2O_2 with Tween 20 for 10 min, followed by 3 washes with sterile water (5 min each); ii) 3% H_2O_2 with Tween 20 for 5 min, followed by 3 sterile water washes, incubation in 70% ethanol for 1 min, 3 sterile water washes, 3.5% bleach for 10 min, and 3 final sterile water washes; iii) 70% ethanol for 1 min, followed by 3 sterile water washes, incubation in 30% H_2O_2 with Tween 20 for 10 min, 3 sterile water washes, 3.5% bleach for 10 min, and 3 final sterile water washes..

-Set 2 treatments: short disinfection in 3% H_2O_2, followed by 2) two methods of scarification, 3) one month of stratification at 4°C, 4) sterilization using method i) from set 1 treatments, and 5) cultivation in the same media as described in set 1 treatments. The same procedure was also performed with a modification where scarification followed stratification.

Scarification. Immersion of seeds for 2 min in conc. H_2SO_4 followed by sterile water washes, or in water at 90°C, followed by leaving the seeds to cool to room temperature while still immersed overnight.

Experiment 2

Seeds underwent neither scarification nor stratification processes. They were rinsed under running water for 5 min, then sterilized following protocol i) from set 1 treatments. After sterilization, whole zygotic embryos were excised and placed horizontally on the bud induction media as described in set 1 treatments. To evaluate and compare the percentage of bud induction in each treatment, a Kruskal–Wallis non-parametric test was performed.

RESULTS

Initially, we evaluated the effects of stratification, scarification, three sterilization methods, and four culture media (LP, SH, and their half-macronutrient versions: HLP, HSH), each supplemented with 4.4, 22, or 44 µM BA, on bud induction using whole seeds as explants. Stratification at 4°C was tested over periods ranging from 7 to 60 days. Scarification involved either immersing the seeds in H_2SO_4 for 2 min or in water at 90°C until reaching room temperature, applied both before and after stratification. Seeds subjected to stratification exhibited high contamination levels (>90%), while those exposed to scarification showed no contamination or bud induction. A few seeds that survived contamination exhibited some morphological changes, but no shoots were formed (**Figure 1d**).

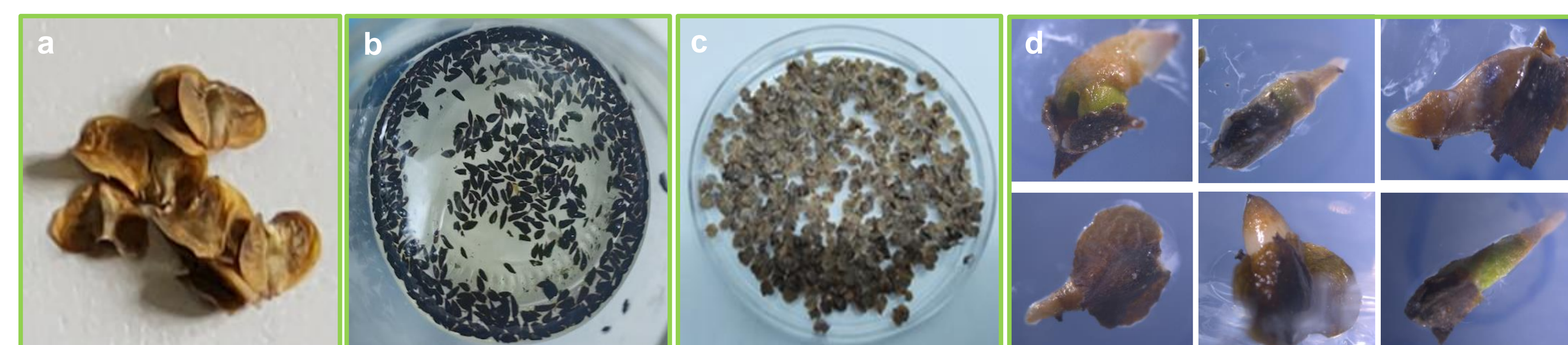

Figure 1. a. Mature seeds of Alerce. **b.** Seeds of Alerce after scarification in concentrated sulfuric acid. **c.** Seeds of Alerce after cold stratification for 30 days. **d.** Seeds of Alerce cultured in different media.

Secondly, an experiment was conducted without stratification or scarification, using whole zygotic embryos as explants and culturing them in the same basal media supplemented with the aforementioned BA concentrations. The contamination rate was 26.7%. Significant differences were observed among treatments ($p = 0.0008$) (**Figure 2**). The highest bud induction was achieved with HSH (72.2 ± 25.4%) and HLP (48.5 ± 8.7%), both supplemented with 4.4 µM BA (**Figure 2**). No bud formation occurred in SH or LP media supplemented with 22 or 44 µM BA (**Figure 2**). Shoots at various stages of elongation are shown in **Figure 3b-d**. Seed viability across all seed lots was 20.1 ± 5.7%, consistent with previous reports for this species.

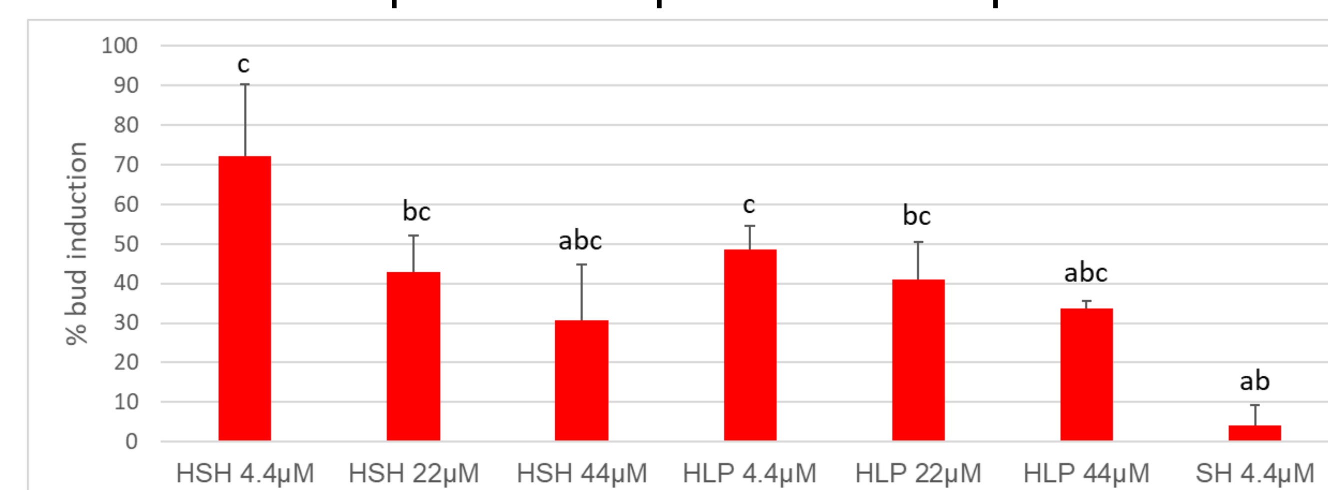

Figure 2. Percentage of bud induction across treatments. Results are expressed in media ± SEM. Different letters indicate statistically significant differences ($p < 0.05$).

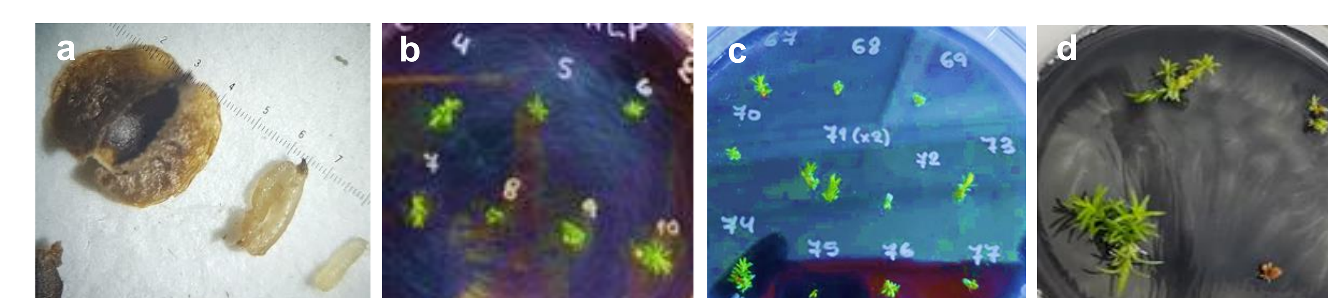

Figure 3. a. Seed, megagametophyte, and zygotic embryo of alerce. **b-d.** Alerce shoots at various stages of elongation.

CONCLUSIONS

The best procedure for bud induction in alerce involves straightforward seed sterilization, without any prior treatment. Zygotic embryos are used as explants and cultured in SH or LP media, using half the macronutrients concentration supplemented with the lowest tested concentration of BA. These findings will aid in developing a propagation protocol through organogenesis for alerce, a threatened species.

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