



In vitro propagation of *Espeletia occidentalis*: a strategy for the conservation and reintroduction of a near-threatened Andean species

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Abstract

Frailejones (*Espeletia* spp.) belong to the Asteraceae family and are endemic to South America. Many species have been severely affected, leading to population declines, including *Espeletia occidentalis* subsp. *antioquiensis*, a Near Threatened species endemic to Colombia. We assessed the feasibility of in vitro mass propagation through organogenesis to support ex situ conservation and potential habitat reintroduction. Specifically, we evaluated the response of apical buds from germinated seedlings culture in Murashige and Skoog (MS) medium supplemented with cytokinins-auxins combinations for shoot induction and root formation. Initially, we observed that seed viability decreased during storage. In germination test, no significant differences were detected between gibberellic acid (GA_3) concentrations; however, a significantly higher germination rate was achieved using moist cotton. The optimal conditions for in vitro multiplication were obtained in MS medium supplemented with 0.05 mg L^{-1} of 6-benzyl amino purine (BAP) and 0.5 mg L^{-1} indole-3-butyric acid (IBA), resulting in an average multiplication coefficient of 2.87 by the third subculture, with no significant differences among culture vessels. The highest rooting response was achieved with 3.0 mg L^{-1} IBA. A substrate mixture of topsoil and coconut fiber resulted in the highest survival rate (76.8%) and promoted subsequent development. This study presents the first report on the seed viability and in vitro propagation potential of *E. occidentalis*, including acclimatization strategies and prospects for habitat reintroduction. Between October 2022 and 2023, approximately 250 plants were successfully transplanted, achieving a 70% survival rate under páramo conditions.

Key message

The escalating threats to the survival of endemic plant species emphasize the urgent need for conservation strategies that integrate ex situ and in situ approaches, along with targeted reintroduction efforts.

Keywords Páramo · *Espeletia* · Conservation · Endemic species · Ecological threats

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Introduction

The páramos are unique ecosystems in the northern Andes Mountain range, home to thousands of endemic species of plants, animals, and other organisms found nowhere else in the world (Rivas Morales 2007; Sarmiento-Pinzón et al. 2013). In Colombia, these ecosystems and their associated wetlands have been designated as strategic areas for biodiversity conservation (Ministerio de Ambiente y Desarrollo Sostenible [MADS] 2002). Their preservation is crucial due to their exceptional biodiversity and the invaluable ecosystem services they provide, particularly in water regulation and climate moderation (Anderson et al. 2011). Plant species play a fundamental role in ecosystem functioning within these environments, as in all terrestrial ecosystems,

by forming the base of the trophic chain and contributing significantly to primary productivity. Therefore, their conservation must be a priority to safeguard biodiversity and ensure the continued provision of ecosystem services to local communities (Castellanos et al. 2010). However, many plant populations face extinction threats, primarily due to habitat destruction, degradation, and the impacts of climate change.

In Colombia, approximately 2.5% of the land area is covered by páramos. These ecosystems are divided into 37 complexes, one of which is the Santa Inés (Belmira) páramo complex, located in the Central Mountain range on the Santa Rosa plateau at an altitude of 3000 to 3400 m above sea level in the department of Antioquia. Within this complex, the frailejón, (*Espeletia occidentalis* subsp. *antioquiensis*) stands out as a species classified as Near Threatened (NT) on the IUCN Red List of Threatened Species. The main threats in this area include the expansion of the agricultural frontier due to pasture establishment for dairy and meat production, as well as the selective exploitation of forest species for timber, firewood, and charcoal (Rivas Morales 2007). Given the increasing pressure on these valuable ecosystems, delimitation programs are being developed to protect them from human activities such as mining and livestock farming, along with the effects of global climate change, which threaten to degrade large areas of these ecosystems at elevations between 3000 and 4300 m above sea level.

The species that inhabit these ecosystems, such as frailejones (*Espeletia* spp.), are highly vulnerable to increasing threats. These plants play a crucial role in regulating and maintaining water sources. In the seven Red Books of Plants, 1,870 species were evaluated, of which 798 (42.6%) were classified under various extinction risk categories, including 88 species of frailejones (Sistema de Información sobre Biodiversidad de Colombia [SIB] 2017). By 2021, 145 frailejón species had been reported, with 90 recorded in Colombia, 55 of which were listed under some threat category (Diazgranados and Castellanos 2021). In June 2022, the Alexander von Humboldt Institute (IAvH) published the latest report on frailejones in Colombia, updating the number of recognized species in the country to 92 (IAvH, 2022).

The frailejones (*Espeletia* spp.), members of the Asteraceae family, are endemic to South America. Conventional in situ conservation methods have not been entirely successful for these species (Berry and Calvo 1989; Tobón 2011; Velasco 2018), highlighting the need for complementary approaches. Domestication and ex situ propagation represent viable strategies to support conservation efforts and promote sustainable use programs. In Colombia, various studies on the genus *Espeletia* have been conducted, encompassing research on ecophysiology, phylogeny, demography, phenology, reproduction, biological control, conservation,

and restoration, among others (Berry and Calvo 1989; Diazgranados 2013; Lara-Figueroa et al. 2017; Rojas-Zamora et al. 2013; Tobón 2011).

Regarding in vitro propagation and conservation, the only available reports come from the Bioplasma research team at Universidad Pedagógica y Tecnológica de Colombia. This team established a micropropagation protocol for *Espeletia paipana* S. Díaz & Pedraza using zygotic embryos from field-collected seeds (Bohórquez-Quintero et al. 2016). The protocol demonstrated its suitability for large-scale propagation programs of this endangered species and serves as a reference for future studies on other species of the genus.

Further research on these species remains crucial, considering their social and biological value, restricted distribution, and the high pressure they face in their natural habitats. Addressing unresolved aspects or areas where progress has been insufficient is essential. These include enhancing seed germination capacity, exploring alternative germination conditions (e.g., different supports and substrates) in both laboratory and nursery settings, reducing basal callus formation during multiplication and rooting phases, improving the direct transfer of in vitro-grown plants to the nursery with higher acclimatization success rates, and studying their adaptive responses to natural páramo conditions.

This study investigates the contribution of in vitro regeneration by organogenesis to the propagation of *Espeletia occidentalis* subsp. *antioquiensis*, for conservation purposes. It focuses on seed germination and viability assessment and the application of plant tissue culture techniques to propagate *Espeletia* through organogenesis in semi-solid culture media. Additionally, the study evaluates the effects of different substrates on plant survival and development under nursery conditions.

Assessing both traditional and in vitro propagation methods of *Espeletia occidentalis* subsp. *antioquiensis* will support its laboratory-scale propagation and potential reintroduction into its natural habitat, potentially enhancing its adaptation to warmer environments projected shortly. These efforts, combined with the engagement of key local community stakeholders through educational initiatives, events, and other conservation strategies, contribute to safeguarding this critical ecosystem and protecting water sources essential for the well-being of all living organisms.

Materials and methods

This study was conducted in the Plant Physiology laboratories at the University of Antioquia and the Plant Growth and Development laboratories of the National University of Colombia, Medellín campus. The acclimatization phase was

performed at the UMAT (Municipal Units of Agricultural Technical Assistance) nursery in Belmira, Colombia.

Plant material

Floral capitula were collected from adult *Espeletia occidentalis* plants in the sectors of La Laguna (Bebedero), Alto de Sabanas, La Cabaña, and Alto de La Gallina, at elevations ranging from 3100 to 3300 m a.s.l., within the Santa Inés páramo, between July 2019 and June 2021. The capitula were sun-dried for three days at approximately 21 °C, with an average humidity of 60% and a 12-h light photoperiod (Lara et al. 2017). The achenes were then extracted, and the number of viable seeds was determined using tetrazolium staining (2,3,5-triphenyl tetrazolium chloride) and in vitro germination assays in cotton and semi-solid culture medium.

Viability test

Viability test by tetrazolium chloride staining

Approximately 100 randomly selected seeds were used in this test (Salazar and Gélvez 2015). Following the methodology proposed by Salazar et al. (2020), a tetrazolium solution was prepared by dissolving the salt in 100 mL of a phosphate buffer solution (1.1% dipotassium phosphate and 0.9% monosodium phosphate). The seeds were immersed in a 1% tetrazolium solution and kept in darkness at a temperature of 21 ± 2 °C for three hours. After this period, the excess tetrazolium was removed by rinsing the seeds three times with deionized water (Espitia et al. 2017). Finally, the seeds were bisected, and the staining pattern was assessed under a stereomicroscope (Olympus model SZ61). The viability percentage was determined based on the categories proposed by Rao et al. (2007), considering only those exhibiting a uniform pink coloration (completely stained) as viable.

Viability test by in vitro seed germination

Disinfection

Seed batches collected in 2019 were packed in muslin bags for handling and surface disinfection using tap water, iodized soap, and a surfactant detergent (Tween™ 20) at 1%, with continuous agitation for 10 min. The seeds were then submerged in a 0.2% carbendazim fungicide solution (Bélico®) combined with 1% Tween™ 20 for 60 min.

Under a laminar flow hood, the seeds were treated sequentially: first, they were submerged in 70% ethanol for 1 min, followed by 1% hydrogen peroxide for 2 min. Subsequently, they were exposed to 3% and 5% NaOCl for 3, 5, and 10 min, as well as 2% and 4% $\text{Ca}(\text{ClO})_2$ for 5, 7, and 10 min. Throughout each step, the seeds were continuously agitated at 80 rpm on a Benchmark Rotomini shaker and rinsed three times with sterile distilled water. Sterile distilled water was used as the control treatment. The disinfection percentage was recorded seven days after sowing.

Germination

The selected seeds for this experiment were then subjected to different concentrations of gibberellic Acid (GA_3) 0.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mg L^{-1} (Espitia Camacho et al. 2017), previously prepared in sterile deionized water. They were then kept under agitation at 80 rpm on a Benchmark Rotomini shaker for one hour. The entire process was carried out in a laminar flow cabinet. Finally, 30 seeds per Petri dish (150 mm $\times$ 15 mm) were sown on absorbent cotton (surgical-grade cotton MK®) with 20 mL of sterile distilled water or in a semi-solid MS/2 culture medium. Each treatment (GA_3 concentration and type of support material) was replicated across ten Petri dishes, using a total of 300 seeds per treatment.

The environment conditions under which the plant material was incubated for in vitro experiments were: a 12-h light photoperiod, 62% relative humidity, a temperature of 21 ± 2 °C, and a light intensity of $46.3 \mu\text{mol m}^{-2} \text{s}^{-1}$. Weekly records were taken, considering germination criteria as radicle emergence, hypocotyl growth, and cotyledon expansion up to 42 days after sowing. The response variables were germination percentage (formula 1), mean germination speed, and mean germination time.

Germination Percentage (GP)

$$= \left(\sum GS \right) / n \times 100 \quad (1)$$

where SG: Germinated seeds. n: Total number of seeds in the trial.

The Mean Germination Rate (MGR, formula 2) was calculated by dividing the final cumulative germination percentage by the total number of days in the trial (Salas, 2013).

Mean Germination Rate

$$= \frac{\% \text{total germination}}{\text{Trial duration}} \quad (2)$$

The Mean Germination Time (MGT) of the analyzed seeds was determined using the formula described by Tomsett and Pritchard (1998) was used (formula 3):

$$\text{Mean Germination Time (MGT)} = \frac{\sum n1 \cdot d1}{N} \quad (3)$$

where n1: Number of seeds germinated on the registration day. d1: Number of days since the beginning of the germination trial. N: Total number of seeds germinated at the end of the trial.

Incubation conditions

The semi-solid culture medium consisted of MS basal salts at half strength (MS/2), supplemented with 30 g L⁻¹ sucrose, 0.5 mg L⁻¹ nicotinic acid, 0.5 mg L⁻¹ pyridoxine, 2 mg L⁻¹ glycine, 1 mg L⁻¹ thiamine, and 3 g L⁻¹ Gellex as the gelling agent, hereafter referred to as Base Culture Medium (BCM). The pH was adjusted to 5.7–5.8 with 1 M HCl and 1 M NaOH and sterilized in an autoclave at 121 °C and 15 psi for 20 min. In vitro plant material was maintained under a 12-h photoperiod, 62% relative humidity, a temperature of 21 ± 2 °C, and a light intensity of 46.3 μmol m⁻² s⁻¹.

Multiplication phase: induction, growth, and development of shoots

Seedlings from germinated seeds, approximately 45 days old, with a height of two to three centimeters, two to four leaves, and no roots, were used. The radicle was removed before planting, as previous trials showed that its presence induced callus formation at the seedling base. The seedlings were established in BCM supplemented with 6-benzylaminopurine (0.0, 0.01, 0.05, and 0.1 mg L⁻¹ BAP) in combination with indole-3-butyric acid (0.0 and 0.5 mg L⁻¹ IBA). Glass flasks (125 mL) containing 25 mL of culture medium were used, and ten consecutive subcultures were performed every 60 days. Each treatment included 30 replicates, with one seedling per culture flask serving as the experimental unit. During the first subculture, the treatment in which both growth regulators promoted the development of plants with morpho-anatomical characteristics similar to those of the mother plants in the field was selected for subsequent in vitro multiplication experiments. The first five subcultures were carried out in 125 mL glass flasks covered with sterile aluminum foil and sealed with parafilm, while the last five were conducted in 1000 mL magenta vessels.

The multiplication coefficient was determined using the following formula (4):

$$\text{Multiplication coefficient} = \frac{\text{Total number of subcultured shoots}}{\text{Initial number of shoots}} \quad (4)$$

Every 60 days, the effect of the growth regulators was evaluated based on the average number of new shoots and leaves per explant, the number and average length of roots per explant, the percentage of callus formation, and the characteristics of the new shoots (e.g., leaf shape, coloration, pubescence, and vitrification, among others).

Rooting phase: effect of growth regulators IBA and NAA on rooting

In vitro seedlings, approximately four centimeters in length, without roots, and with an average of five new leaves, were obtained after 60 days in the multiplication medium (MS/2 supplemented with 0.05 mg L⁻¹ BAP + 0.5 mg L⁻¹ IBA). Subsequently, they were sown in BCM supplemented with IBA (0.0, 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 mg L⁻¹) or 1-naphthaleneacetic acid (NAA: 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg L⁻¹). Glass flasks (125 mL) containing 25 mL of culture medium were used. The effect of the growth regulator was evaluated based on the number and average length of roots per explant, the presence or absence of callus, and the morphological characteristics of the roots and plants after 60 days of cultivation. Each treatment included 30 replicates, with one seedling per culture flask as the experimental unit.

The rooting experiment aimed to identify the most effective treatment for promoting root development and growth. The treatment that resulted in optimal plant development, characterized by a well-formed and functional root system, was selected for subsequent rooting trials. This treatment was then used to cultivate rootless seedlings, which were subsequently transferred to the nursery phase after root formation. Five in vitro seedlings were planted per culture vessel (magenta flasks) to facilitate this process.

Acclimatization phase: establishing a protocol by evaluating different substrates under nursery conditions

In vitro rooted plants with approximately 60 days of development in rooting medium supplemented with 3.0 mg L⁻¹ IBA were used. The plants were transferred in culture vessels (magenta flasks) to the nursery in the municipality of Belmira and kept under pre-adaptation conditions for 15 days. Before transplantation into different substrates, the plants were individualized, and their roots were washed with tap water to remove culture medium residues. They were then planted in 50-cell polypropylene trays (55 × 29

× 12 cm) preserving their roots. To maintain humidity, the plants were covered with transparent plastics for 15 days; after which small perforations were made to regulate relative humidity and improve aeration. Following the pre-adaptation phase, the covers were removed after two weeks, exposing the plants to the nursery's environmental conditions.

The evaluated substrates included topsoil (T1), coconut fiber substrate (T2), a mixture of topsoil and coconut fiber substrate in a 3:1 ratio (T3), and the nursery substrate (T4), composed of sand, chicken manure, black soil, and rice husk in a 7:1:1:1 ratio. A physicochemical analysis was performed on each substrate at the Soil Laboratory of the National University of Colombia, Medellín campus. None of the substrates underwent sterilization to preserve the native microbiota and prevent alterations in the physicochemical properties essential for plant development. Plants grown in the nursery were kept at an average temperature of 22 °C, 62% relative humidity, and a light intensity of 344 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Manual irrigation was applied weekly for 90 days. Each experimental unit comprised five trays containing 25 plants, totaling 125 plants per treatment. The number of surviving plants in each treatment was recorded weekly to quantify the survival rate at 90 days post-transplantation.

Data analysis

The experiments followed a completely randomized design, and the results were subjected to the normality assumptions test using the Kolmogorov–Smirnov test, the Shapiro–Wilk test, or the Levene test, depending on the case. Data were analyzed through an individualized analysis of variance (ANOVA) for each variable with a 95% confidence level, followed by paired comparisons using Tukey's test. When the normality assumption was not met, the non-parametric Kruskal–Wallis or Wilcoxon test was applied, and differences between treatments were assessed using Dunn's test. All statistical analyses were performed using the StatR and PlotsR applications of the RWizard program (Guisande et al. 2014) with R packages (R Development Core Team 2015).

Results

Viability test

Seed viability by staining with Tetrazolium

Based on observations from field visits and surveys, flowering was found to occur between March and April in a non-synchronous manner, with mature floral capitula appearing

from May onward. Fruiting and seed dispersal took place between June and September, with peak floral senescence recorded between October and November. Given this phenological pattern, floral capitula were collected in September 2019, yielding 4311 seeds from 8613 achenes, with 50.05% achenes containing seeds and viability of 63% according to the tetrazolium test. In June 2021, a total of 8349 achenes were collected from which 4257 seeds were extracted, resulting in 50.98% of achenes containing seeds. These seeds were stored at 5 °C, and a tetrazolium viability test was conducted over nine consecutive months. The average viability of this batch was 28.20%, confirming a decline in seed viability during storage. Therefore, immediate sowing after collection is recommended. These findings provide estimates of viability percentages for short and long-term storage and future experimental trials.

Seed viability by in vitro germination test

Disinfection

The initial seed disinfection process conducted outside the laminar flow hood, was effective, as no contamination was observed in any treatment replicates, including the control, achieving 100% surface sterilization. Regarding the effect of disinfectants, prolonged exposure times and higher concentrations of $\text{Ca}(\text{ClO})_2$, led to tissue darkening and damage, whereas NaOCl did not produce these adverse effects.

Germination

Regarding viability through germination, of the 3300 seeds collected in 2019 and sown on cotton or semi-solid culture medium, a total germination rate of 42.60% (1406 seeds) and a seedling survival rate of 86.2% (1212 seedlings) were recorded. Seeds sown on cotton began germination on the fifth day across all evaluated GA_3 concentrations, whereas those sown on semi-solid culture medium initiated germination from day 14 at all GA_3 concentrations. Although the highest germination rates were observed at 8.0 mg L^{-1} GA_3 , no significant differences were found compared to the control treatment (0.0 mg L^{-1} GA_3). In the semi-solid MS/2 culture medium, germination rates were lower, occurring predominantly in two phases (days 14 and 28) across most GA_3 concentrations.

No significant differences were found between treatments or supports regarding mean germination time ($p > 0.05$). However, significant differences were observed between the two supports in terms of germination percentage and rate ($p < 0.05$), with the highest values recorded on cotton. For the 8.0 mg L^{-1} GA_3 treatment on cotton resulted in the highest

Table 1 Adjusted mean of analyzed variables for each GA₃ treatment (0.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mg L⁻¹) and support type (Cotton and Semi-solid Medium)

Adjusted mean				
Support type	Gibberellin mg L ⁻¹	Cumulative germination percentage	Σ MGR	Cumulative MGT
Cotton	0.0	48.48 ^a	2.75 ^a	20.95 ^a
	2.0	42.33 ^a	2.38 ^a	20.00 ^a
	4.0	34.66 ^a	2.07 ^a	19.25 ^a
	6.0	37.22 ^a	2.38 ^a	18.17 ^a
	8.0	55.00 ^a	3.11 ^a	20.32 ^a
	10.0	49.69 ^a	2.59 ^a	20.95 ^a
	0.0	17.00 ^b	0.92 ^b	20.86 ^a
Semi-solid culture medium	2.0	20.83 ^b	1.17 ^b	20.02 ^a
	4.0	14.81 ^b	0.72 ^b	23.27 ^a
	6.0	20.00 ^b	0.92 ^b	24.37 ^a
	8.0	18.14 ^b	0.86 ^b	24.00 ^a
	10.0	14.07 ^b	0.67 ^b	23.39 ^a

*Means with different letters indicate significant differences between treatments for the analyzed variable ($p < 0.05$)

germination percentage (55%) and a mean germination rate of 3.11 seeds/day (Table 1).

Multiplication phase: induction, growth, and development of shoots

In all treatments, shoot differentiation was achieved; however, morphological variations were observed depending on the type and combination of growth regulators. In treatments **T1** (free of growth regulators), **T2** (0.0 mg L⁻¹ BAP + 0.5 mg L⁻¹ IBA), **T3** (0.01 mg L⁻¹ BAP + 0.0 mg L⁻¹ IBA), and **T4** (0.01 mg L⁻¹ BAP + 0.5 mg L⁻¹ IBA), the characteristic rosette growth of the species did not occur. Instead, both the stem and leaves elongated, and the leaves exhibited minimal or no pubescence. In contrast, in treatments **T5** (0.05 mg L⁻¹ BAP + 0.0 mg L⁻¹ IBA), **T6** (0.05 mg L⁻¹ BAP + 0.5 mg L⁻¹ IBA), **T7** (0.1 mg L⁻¹ BAP + 0.0 mg L⁻¹ IBA), and **T8** (0.1 mg L⁻¹ BAP + 0.5 mg L⁻¹ IBA), the vitroplants exhibited a rosette growth pattern similar to that of in situ páramo plants. Additionally, T5, T7, and T8 the leaves were, thick, brittle (vitrified leaves), deformed, and exhibited little or no pubescence. Moreover, multiple shoots formed, making them difficult to count and separate. In contrast, treatment six exhibited leaves without malformations, light green coloration, and noticeable pubescence (Fig. 1). Regarding callus formation at the plant base, treatments T1 to T5 did not induce callus formation. In contrast, plants in T8 developed abundant callus, followed by T6 and T7, which showed minimal callus formation. The interaction between the two



Fig. 1 Morphological traits of 60-day-old in vitro-grown *E. occidentalis* plants in response to the multiplication treatments studied. **T1** (No growth regulators), **T2** (0.0 mg L⁻¹ BAP + 0.5 mg L⁻¹ IBA), **T3** (0.01 mg L⁻¹ BAP + 0.0 mg L⁻¹ IBA), **T4** (0.01 mg L⁻¹ BAP + 0.5 mg L⁻¹

IBA), **T5** (0.05 mg L⁻¹ BAP + 0.0 mg L⁻¹ IBA), **T6** (0.05 mg L⁻¹ BAP + 0.5 mg L⁻¹ IBA), **T7** (0.1 mg L⁻¹ BAP + 0.0 mg L⁻¹ IBA), and **T8** (0.1 mg L⁻¹ BAP + 0.5 mg L⁻¹ IBA)

Fig. 2 Effect of the growth regulators BAP and IBA on the mean shoot number per *E. occidentalis* explant after 60 days of cultivation. Different letters indicate statistically significant differences between treatments for this variable ($p = 0.0263$)

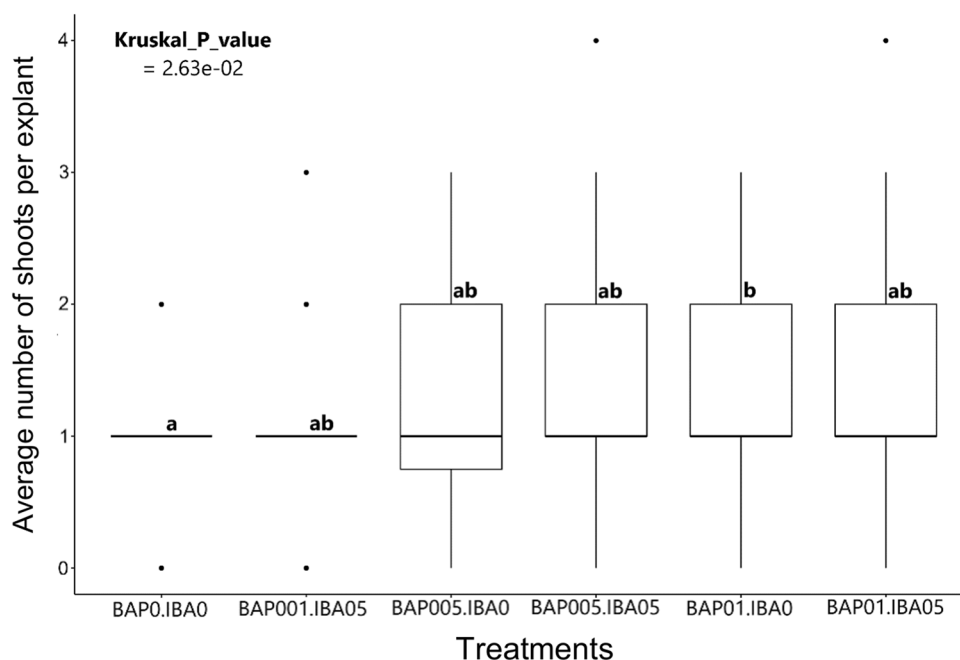
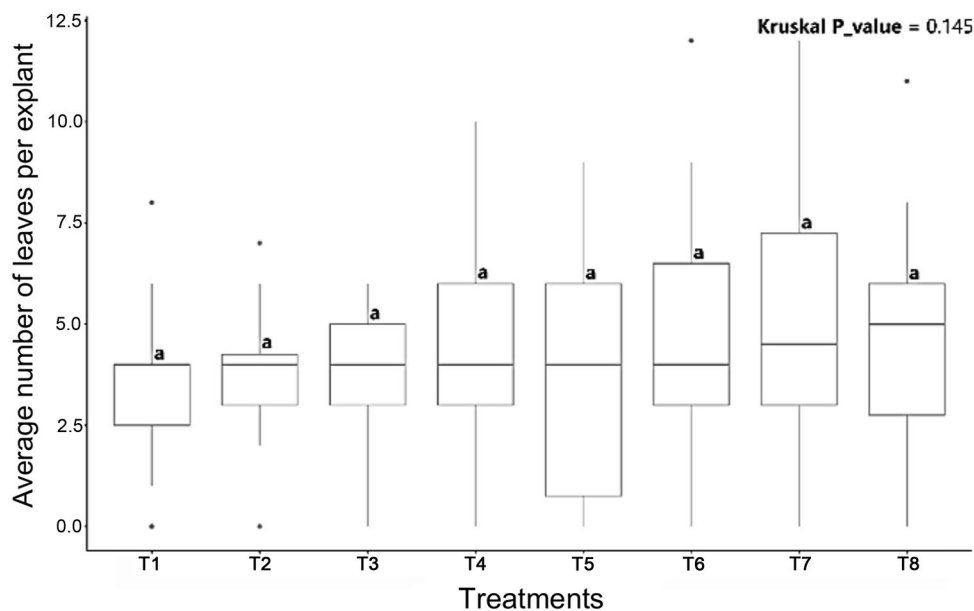


Fig. 3 Effect of the interaction between the growth regulators BAP and IBA on the mean leaf number per *E. occidentalis* explant after 60 days of cultivation. Identical letters indicate no statistically significant differences between treatments for this variable ($p = 0.145$)

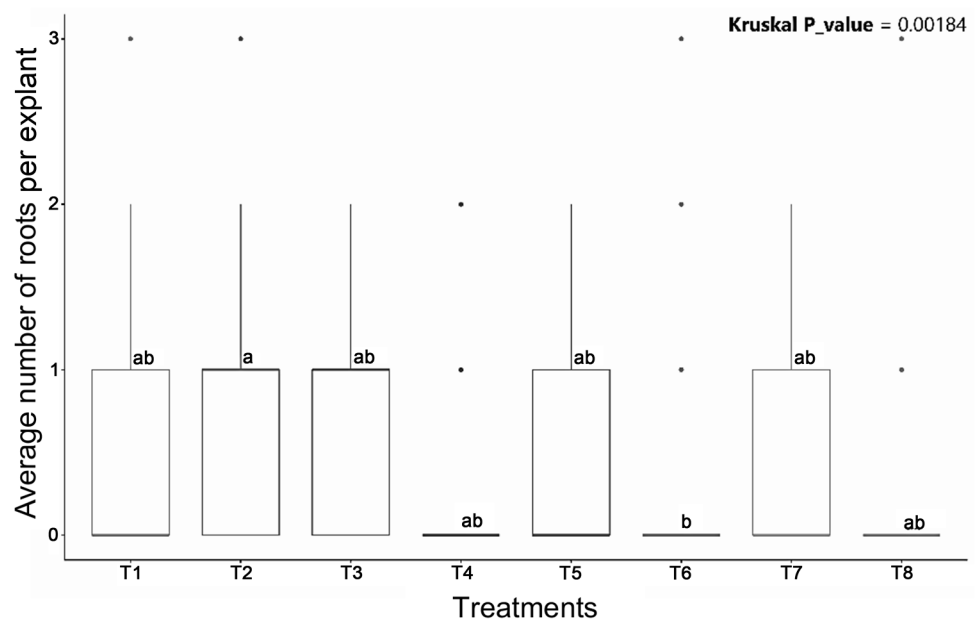


growth regulators was significant ($p = 0.008$), with callus formation being more pronounced in plants treated with 0.5 mg L^{-1} IBA and increasing proportionally with higher BAP concentrations. Therefore, both growth regulators played a key role in callus induction.

The Kruskal–Wallis non-parametric test revealed significant differences in the number of shoots for BAP concentrations ($p = 0.00765$), but no significant differences were found between IBA concentrations ($p = 0.573$) regarding the interaction between both factors. Significant differences were observed for the number of shoots ($p = 0.0263$, Fig. 2). For the number of leaves, significant differences were found ($p = 0.0297$), with an increase in the number of leaves per

shoot as the BAP concentration increased. In contrast, no significant differences were observed for IBA concentrations ($p = 0.309$) nor the interaction with BAP ($p = 0.145$, Fig. 3). Regarding the number of roots, significant differences were found among BAP concentrations ($p = 0.0112$), with a clear decrease in the number of roots at higher BAP concentrations. Significant differences were also observed for IBA concentrations ($p = 0.00845$) as well as for the interaction between both factors ($p = 0.00184$, Fig. 4).

Fig. 4 Effect of the interaction between the growth regulators BAP and IBA on the mean root number per *E. occidentalis* explant after 60 days of cultivation. Different letters indicate statistically significant differences between treatments for this variable ($p = 0.00184$)



Effect of subculture number on in vitro multiplication of *E. occidentalis*

Based on the results of the multiplication experiment, the treatment containing 0.05 mg L^{-1} BAP and 0.5 mg L^{-1} IBA was selected to carry out the propagation process and monitor the behavior of the plants in terms of the conservation of key frailejon characteristics (rosette growth, oval and pubescent leaves, and light green coloration) as well as the multiplication coefficient (MC) over ten subcultures. The number of subcultures affected the MC, with an increase observed until the third subculture (2.475), followed by a decline to 1.0 in the fifth subculture. When the culture vessels were replaced with magenta flasks, the MC rose again (3.263), but in subsequent subcultures, it gradually decreased, reaching an average of 1.1 in the tenth subculture. The average multiplication coefficients were 1.89 ± 0.86 for magenta flasks and 1.70 ± 0.64 for glass flasks. No significant differences were found between the two culture vessels in terms of MC ($p < 0.05$).

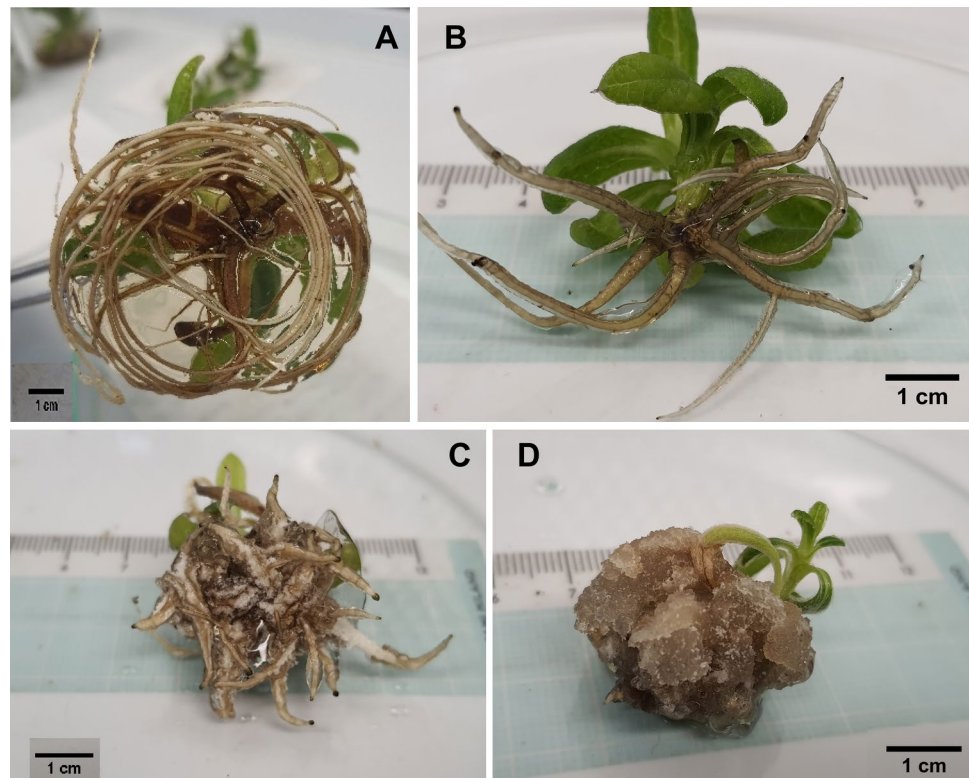
Rooting phase: effect of growth regulators IBA and NAA on rooting

Each treatment evaluated in this phase promoted the emergence of new roots, starting from the third week of planting. In the presence of the growth regulator IBA, the initial roots appeared thin, whereas those in the medium containing NAA formed thick and brittle root structures from the outset. Callus formation at the base of the plants did not interfere with root development when IBA was used. However, in the presence of NAA, callus formation inhibited root growth and development. Notably, as the concentration of

the growth regulator increased, both the number and average length of roots increased, alongside the formation of callus, with over 40% of explants exhibiting callus at treatment seven (4.0 mg L^{-1} IBA). Treatment six (3.0 mg L^{-1} IBA) yielded the best response, with the highest average number of roots (4.86) and the greatest root length (5.31 cm), without callus formation at the base of the plants. Additionally, in this treatment, roots exhibited desirable characteristics: thin, elongated, and with the presence of absorbent hairs. In contrast, with NAA, low concentrations led to a higher presence of callus, which increased significantly in treatment T13 (0.2 mg L^{-1} ; Fig. 5C, D).

The Kruskal–Wallis non-parametric test revealed significant differences in both the number and average length of roots per plant among the ten IBA treatments ($p < 0.001$). Starting from T6 (3.0 mg L^{-1} IBA), a greater number and average length of roots were observed, with treatments T10 (7.0 mg L^{-1} IBA) and T9 (6.0 mg L^{-1} IBA) exhibiting the highest values. However, as callus formation increased in the explants from T7, treatment T6 was selected for further analysis, as it demonstrated a positive relationship between root number and a lower percentage of callus. Significant differences were also found in the number of roots among the NAA treatments ($p < 0.001$). The highest average number of roots was achieved in treatment T14 (0.3 mg L^{-1}), followed by T16 (0.5 mg L^{-1}). The average number of roots increased from T12 (0.1 mg L^{-1}), but this was accompanied by an increase in callus presence in the explants, surpassing 30% (Fig. 6).

Fig. 5 Effect of IBA and NAA concentrations on root and callus induction, as well as their characteristics, in *E. occidentalis* plants grown in vitro after 60 days of cultivation: a comparative analysis of root number, root length, and callus formation frequency. (A and B) Roots exhibiting desirable characteristics: thin, elongated, and with absorbent hairs. (C and D) Root formation interference due to callus presence, resulting in reduced root growth and development



Acclimatization phase

Evaluation of different substrates in nursery conditions

In the first three treatments, notable improvements in the morpho-anatomical characteristics of the plants were observed. These included an increase in leaf area, the formation of new leaves, and a shift in coloration from dark green to light green, accompanied by a more prominent presence of trichomes. Furthermore, a well-developed root system with the presence of absorbent hairs was evident at the end of the 90-day hardening period. Conversely, in treatment four, foliar and root deterioration was observed, marked by necrosis and rot affecting both the leaves and roots.

Survival percentage

Analysis of survival rates revealed significant differences in the number of live plants per treatment. Treatment three, consisting of a mixture of topsoil and coconut fiber substrate (Fig. 7C), achieved the highest survival percentage at 76.8%. This was followed by treatment two, which used coconut fiber substrate (57.6%; Fig. 7B), and treatment one, which used topsoil (52%; Fig. 7A). In contrast, treatment four, utilizing a nursery mixture, resulted in only 2.4% survival (97.6% mortality; Fig. 7D).

The hardened plants were then transferred to plastic bags (15 × 20 cm), where they remained for 60 days before being

transplanted into the Santa Inés páramo, specifically in the Alto de Sabanas area. After 12 months, the survival rates averaged approximately 70% (Fig. 7E, F, G).

Discussion

Observations conducted between 2019 and 2022 provided valuable insights into the reproductive periods of *E. occidentalis*, which could guide future studies aimed at precisely determining the optimal timing for seed collection through detailed and systematic phenological monitoring. Flowering began between March and April, followed by fruiting and dispersal from June to September. This finding contrasts with the reproductive cycle described for *Espeletia grandiflora* Humb. and Bonpl., (Lara et al. 2017; Tobón-Monroy 2011), where flowering, fruit production, and the initiation of dispersal were observed during the latter half of the year (September to November), with most individuals showing senescent inflorescences.

Additionally, seed quantification results revealed that the percentage of achenes containing seeds varied across different years and months evaluated. This variation may be linked to fluctuations in abiotic factors influencing plant development, as outlined by Yepes and Silveira (2011). Other studies have highlighted fluctuations in seed production over different years, attributed to biotic factors such as herbivory and damage caused by pathogens (Girado-Beltrán

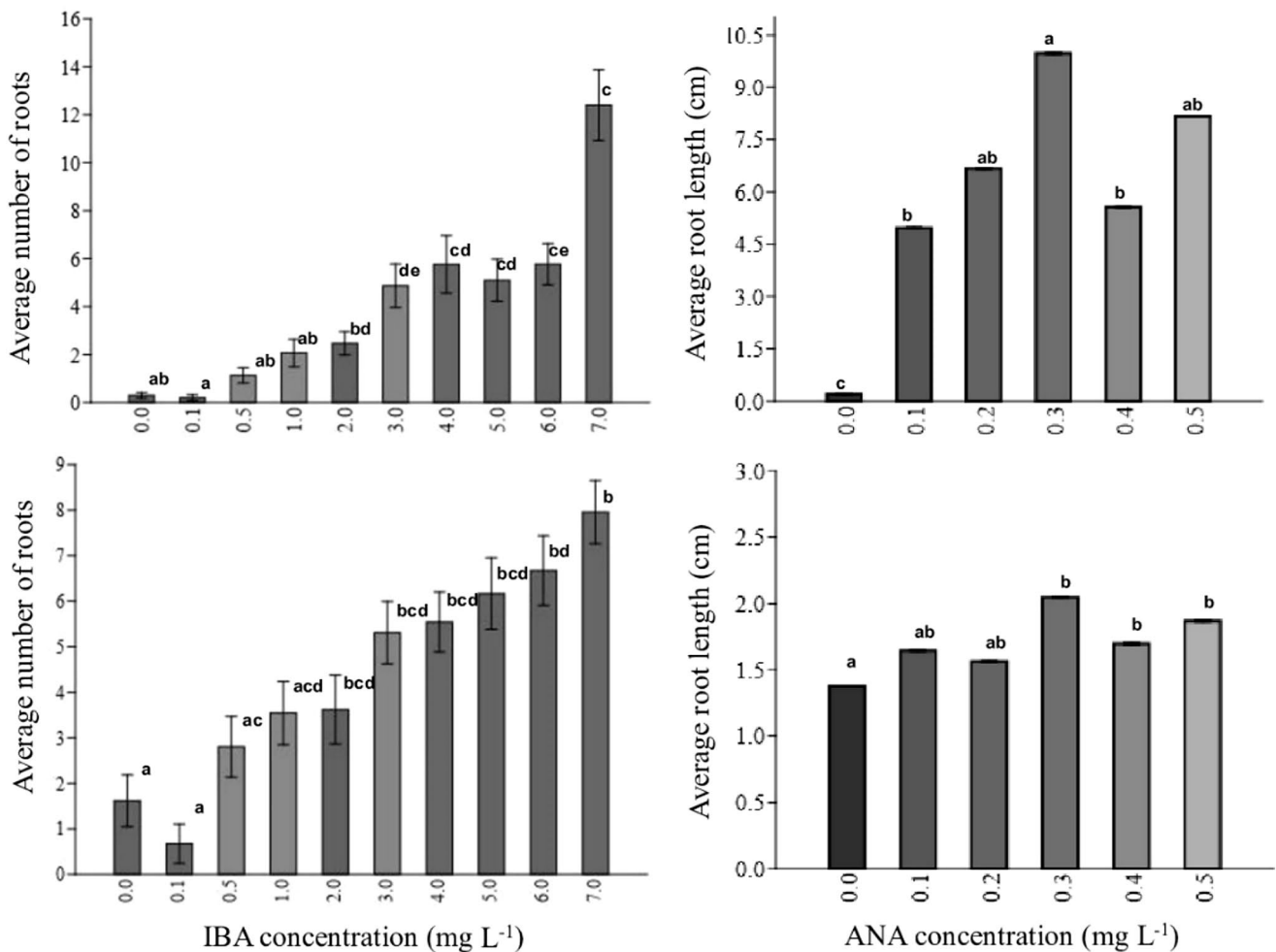


Fig. 6 Formation, growth, and development of *E. occidentalis* roots after eight weeks of rooting treatments. (A) Mean root number per explant and mean root length in treatments with IBA (T1: 0.0; T2: 0.1; T3: 0.5; T4: 1.0; T5: 2.0; T6: 3.0; T7: 4.0; T8: 5.0; T9: 6.0, and T10: 7.0 mg L⁻¹). (B) Mean root number per explant and mean root length

in treatments with NAA (T1:0.0; T2: 0.1; T3: 0.2; T4: 0.3; T5: 0.4 and T6: 0.5 mg L⁻¹). Error bars represent the standard deviation (SD). Different lowercase letters above the error bars indicate statistically significant differences between treatments for the analyzed variable ($p < 0.05$)

et al. 2015). For *E. paipana*, Bohórquez-Quintero et al. (2016) reported that 69.27% of achenes contained seeds, of which 60.5% were deemed suitable for cultivation (4370 seeds). These findings slightly differ from those observed in *E. occidentalis*, where, between 2019 and 2022, an average of 58.04% of achenes containing seeds of healthy appearance, free of superficial damage, and deemed suitable for viability testing. In *E. curialensis*, Velasco (2018) reported that less than 5% of achenes contained developed seeds, which was attributed to herbivory damage or insufficient embryo maturation. Lara et al. (2017) reported the collection of 3270 achenes, of which 36.8% contained seeds. While climatic factors play a role in altering and modifying phenological processes, understanding this behavior in *E. occidentalis* highlights that comparisons with other species based solely on climate data may not lead to general conclusions. It is therefore recommended to conduct

species-specific studies that also consider other factors, such as self-pollination, pollinators, and interspecific relationships. To better comprehend the phenological patterns and other biotic and abiotic factors affecting *Espeletia* species, it is crucial to continue research to gather valuable information that can inform conservation strategies and help protect the available biodiversity.

Different disinfection protocols for in vitro sowing of achenes and frailejon seeds report the use of sodium hypochlorite and calcium hypochlorite as the main disinfecting agents (Cárdenas-Burgos et al. 2019; Chun et al. 1997; Pérez-Nuñez 2020; Ruiz et al. 2020; Quezada and Almanza 2023). Disinfection responses with NaOCl are typically around 90% (Rache and Pacheco 2009; Ramírez-Rengifo et al. 2011), while with Ca(ClO)₂, these percentages range between 64 and 94%. Disinfectant concentrations in these studies generally vary from 1 to 4%, with immersion times



Fig. 7 Plant response at 90 days after planting under nursery conditions across different treatments. **(A)** Capote soil. **(B)** Coconut fiber substrate. **(C)** Mixture of capote soil and coconut fiber substrate (3:1). **(D)** substrate used in the Belmira nursery (sand, chicken manure, black soil, and rice husk in a ratio of 7:1:1:1). **(E)** Plants of 60 days of growth

and development in plastic bags. **(F)** Municipal staff from Belmira conducting planting in the páramo ecosystem. **(G)** Plants adapted to the páramo ecosystem 12 months after planting in the Alto de Sabanas sector

ranging from five to ten minutes (Araque-Barrera et al. 2016; Bohórquez-Quintero et al. 2016).

The results of this research contrast with those of previous studies, as the prior disinfection protocol, performed in the laminar flow hood, included biocidal agents (Echenique and Huanca 2022), which were sufficient to achieve 100% surface disinfection. The application of $\text{Ca}(\text{ClO})_2$ induced tissue darkening and, in some cases, damage that affected cell viability and regenerative capacity. This highlights the importance of carefully selecting the disinfectant, optimal

concentration, and exposure time to avoid seed damage and minimize the impact on the organogenic response, as described by Campos et al. (2020), Díaz-Lezcano et al. (2023) and Gonçalves-Magalhães et al. (2023).

The evaluation of seed viability using the 1% tetrazolium topographic test revealed significant variability across the different periods (2019 to 2021). The observed trends were consistent with those reported by Bohórquez-Quintero et al. (2016) for seeds of *E. paipana*, Mancipe et al. (2018) for four *Espeletia* species between 2014 and 2015, Mancipe-Murillo

(2020) for seeds collected in 2017, and Velasco (2018) for *Espeletia curialensis* from individuals at three different elevations within El Cocuy National Natural Park. The variation in seed viability can be attributed to factors such as seed age, storage conditions, and climatic conditions during collection. Additionally, test conditions, including tetrazolium concentration and exposure time, may also influence the results (Hernanz et al. 2005). Therefore, it is crucial to consider each plant species-specific characteristics and adjust the test parameters accordingly to obtain accurate and reliable seed viability evaluations. This method can be particularly useful for monitoring the viability of seeds stored over extended periods (Hadi et al. 2023; Ribeiro et al. 2010). Given that *E. occidentalis* is an endemic species, the tetrazolium viability test provides valuable information regarding the proportion of seeds capable of contributing to the establishment of new plants, an essential consideration for the species' propagation and conservation efforts.

Regarding the evaluation of seeds viability through the germination test, gibberellins, such as GA_3 , have been commonly used to break dormancy and promote germination (Baskin & Baskin 2004; Perez-Martinez et al. 2014). However, this study did not find statistically significant differences in germination percentages across the evaluated GA_3 concentrations. In contrast, Bohórquez-Quintero et al. (2016) reported 50% germination in *E. paipana* when cultured in MS/4 medium supplemented with 1 mg/L of GA_3 . In the same culture medium, *Espeletiopsis rabanalensis* showed an 8.2% germination percentage, with only 3.58% of normal-looking seedlings obtained (Araque-Barrera et al. 2016). Ramírez-Cabrera (2014) reported a germination percentage between 6% and 13.5% in *Espeletiopsis corymbosa* seeds sown in MS after imbibition in a 200 mg L⁻¹ GA_3 solution for 24 h, with the highest germination observed in the treatment without the growth regulator. This finding aligns with that of Rache and Pacheco (2009) in *Espeletiopsis muiska*, where 91% germination was achieved in MS medium without growth regulators. According to Bewley (1997) and López et al. (2018), not all seeds require an external stimulus from growth regulators to germinate; in the case of frailejones, the seeds may contain sufficient endogenous levels of these regulators to initiate and complete the germination process without external supplementation. Based on the results obtained for *E. occidentalis*, it can be proposed that its seeds respond to the presence of water without the need for additional GA_3 . As described by Obroucheva et al. (2017) and Saldivar-Igñesis et al. (2010), water plays a critical role as a medium that triggers metabolic processes in reserve substances (carbohydrates, lipids, proteins, etc.) that lead to germination (Araujo et al. 2016). This was evident in the moistened cotton, which maintained and facilitated rapid and efficient water absorption to initiate

the germination process. In the semi-solid culture medium with half-strength salts, the water potential was higher than in the cotton substrate, which reduced water availability for the seeds and delayed germination. Given the limited literature on *Espeletia* that comprehensively discusses germination-associated parameters, further studies are essential to better understand their behavior. This will help establish strategies to increase germination speed and quantity while reducing germination time, particularly considering vigor aspects, as discussed in studies on *Heliophilous* species by Quiroga (2016).

Regarding the multiplication stage, the treatment with 0.05 mg L⁻¹ BAP plus 0.5 mg L⁻¹ IBA was selected to continue the micropropagation process, as it preserved the morpho-anatomical characteristics of plants in their natural habitat. Studies on related species, *Espeletiopsis rabanalensis* and *E. paipana*, concur that this combination of growth regulators is the most suitable for the multiplication stage (Araque-Barrera et al. 2016; Bohórquez-Quintero et al. 2016). Similarly, Rache and Pacheco (2009) determined that 0.5 mg L⁻¹ IBA and 0.01 mg L⁻¹ BAP were optimal for shoot proliferation in *Espeletiopsis muiska*. These findings reaffirm the critical role of the balance between growth regulators in promoting explant growth and development (Bahadur et al. 2015; Wu et al. 2023; Singh 2015). However, they contrast with the classical model proposed by Miller and Skoog (1953), which states that organogenesis is influenced by the auxin-cytokinin ratio (A/C), where higher cytokinin concentrations relative to auxins promote shoot formation. Nevertheless, considering the potential contribution of endogenous growth regulators, the low exogenous BAP concentration used in this study may have been sufficient to induce shoot formation.

In this study, as observed in previously described *Espeletia* species, the auxin-to-cytokinin ratio (A/C) was higher for IBA, and shoot formation occurred in all treatments, including the control. Although IBA is commonly used to promote root development, some studies suggest that higher concentrations may also induce shoot formation and facilitate apical meristem differentiation (Francisco-Rodríguez et al. 2025; Zhang et al. 2022), emphasizing the complexity of growth regulator interactions. During this phase, the multiplication coefficient (MC) was monitored every 60 days over ten consecutive subcultures, with values ranging from 1.1 to 3.26. These results are consistent with those reported by Bohórquez-Quintero et al. (2016) for *E. paipana*. However, MC values declined as the number of subcultures increased, potentially due to changes in morphogenesis potential, which may have limited the capacity for active cell division and proliferation (dos Reis de Oliveira 2022; Graner et al. 2019).

During the rooting phase, thin, elongated roots with absorbent hairs developed under different IBA concentrations, supporting normal *in vitro* plants growth and development. This response is consistent with the findings of Araque-Barrera et al. (2016), who reported that a concentration of 3.0 mg L^{-1} IBA promoted optimal root development for their study. Similarly, Bohórquez-Quintero et al. (2016) and Rache and Pacheco (2009) observed improved rooting responses at 5.0 mg L^{-1} and 0.01 mg L^{-1} IBA, respectively. Considering the role of auxins in adventitious root formation (Deltalab et al. 2024; Guan et al. 2019), the variation in responses may depend on the interaction between auxins and specific receptors involved in the rooting process, which is species-dependent (Olatunji et al. 2017). The enhanced response to IBA compared to ANA aligns with the findings of Uribe et al. (2012), who associated the low root formation and increased callus production in the presence of ANA with inhibitory effects. These effects may be linked to ANA's greater stability in the culture medium, resulting from its lower susceptibility to enzymatic degradation or breakdown by environmental factors such as heat and photo-oxidation (Garay-Arroyo et al. 2014; Taiz and Zeiger 2002). In this study, the inhibitory effect of ANA persisted even at low concentrations (0.0 and 0.5 mg L^{-1}), whereas 3.0 mg L^{-1} IBA significantly promoted root formation. This response is likely attributed to the rapid metabolism of IBA, which may necessitate higher concentrations to achieve optimal rooting. The effectiveness of adventitious root formation is closely linked to the type of auxin, its concentrations, and tissue's physiological sensitivity.

In the final propagation stage, hardening, the highest survival rates were observed in topsoil (forest humus) alone or mixed with soil, consistent with findings reported by Araque-Barrera et al. (2016), Bohórquez-Quintero et al. (2016), and Rache and Pacheco (2009). Physicochemical analyses of the substrates (Data not shown) revealed that the coconut substrate and the topsoil (forest humus)-coconut exhibited favorable chemical (nutrient balance) and physical (water retention capacity, drainage, and aeration) properties, facilitating plant adaptation, growth, and functionality without the need for fertilizers. These results demonstrate that *in vitro*-derived plants possessed the necessary vigor and physiological traits for successful hardening. Despite significant aluminum saturation in the substrate mixture (6.9%), the plants showed no signs of toxicity, likely due to their inherent tolerance to high-aluminum páramo soil, as previously described for *Espeletia hartwegiana* by Tintinango (2019).

Conclusion

This study represents the first comprehensive report on the *in vitro* propagation of the endemic *Espeletia occidentalis* through organogenesis, providing a detailed protocol encompassing seed viability assessment, shoot proliferation, rooting, nursery acclimatization, and initial reintroduction into its natural habitat. Significant advancements were made in key areas, including the characterization of flowering and fruiting periods, the impact of collection time and storage duration on seed viability, and the enhancement of *ex situ* germination rates in an inert substrate. These findings contribute substantially to the conservation strategies for *E. occidentalis* and establish a foundation for the development and application of similar propagation approaches for other *Espeletia* species facing different extinction risk categories. Furthermore, this research supports the restoration of páramo ecosystems, which are of critical ecological importance to Colombia, reinforcing the role of biotechnological tools in the conservation and sustainable management of high-mountain plant species.

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Data availability The raw data supporting this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no relevant financial or non-financial conflicts of interest. Additionally, they have no financial or proprietary interests in any material discussed in this article.

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