ARTÍCULO DE INVESTIGACIÓN

In vitro propagation of *Salvia Pamplonitana*: a critically endangered and endemic species of Colombia

Propagación *in vitro* de *Salvia Pamplonitana*: una especie endémica y en peligro crítico de Colombia

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ABSTRACT

This study aimed to develop an *in vitro* regeneration protocol for the propagation and conservation of critically endangered species such as *Salvia pamplonitana*, an endemic plant of Colombia exclusive to the Pamplona Community. Despite its ornamental potential, *S. pamplonitana* faces critical endangerment due to habitat destruction caused by human activities such as deforestation, urbanization, and agricultural expansion in densely populated regions. This research addressed the absence of biotechnological studies on *S. pamplonitana* by developing a methodology to promote *in vitro* germination and micropropagation, alongside contamination control measures for seed capsules and nodal explants. Successful regeneration of *S. pamplonitana* was achieved in *in vitro* from adult plants using nodal segments and seeds collected from a single individual in the University of Pamplona's greenhouse. Surface disinfection treatments for seed capsules demonstrated the significant impact of both NaOCl concentration and exposure duration on seed contamination and germination rates. Furthermore, the evaluation of surface disinfection treatments on nodal segments effectively managed contamination and oxidation levels, highlighting the influence of exposure duration to fungicide, isodine®, and alcohol, with higher concentrations and longer exposure times resulting in increased oxidation and reduced contamination, while emphasizing the widespread use of sodium hypochlorite for surface sterilization. Optimal shoot proliferation was observed with nodal explants on Murashige and Skoog (MS) medium supplemented with 2.0 mg/l^{1} (8.8 µM) 6-Benzylaminopurine and 0.05 mg/l^{-1} (0.14 µM) gibberellic acid (GA_3), while rooting was successful on MS medium supplemented with 3.0 mg/l^{-1} (14.7 µM) indole butyric acid. The rooted plantlets were acclimatized to *ex vitro* conditions. Additionally, the study demonstrated enhanced germination through scarification treatment and absence of light, offering potential benefits for *ex situ* preservation, restoration, and reestablishment of *S. pamplonitana* populations in their natural habitat.

Keywords: *Salvia pamplonitana*; critically endangered; *in vitro* regeneration.

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RESUMEN

Este estudio tuvo como objetivo desarrollar un protocolo de regeneración *in vitro* para la propagación y conservación de especies en peligro crítico como *Salvia pamplonitana*, una planta endémica de Colombia exclusiva de la Comunidad de Pamplona. A pesar de su potencial ornamental, *S. pamplonitana* enfrenta un peligro crítico debido a la destrucción del hábitat causada por actividades humanas como la deforestación, urbanización y expansión agrícola en regiones densamente pobladas. Esta investigación abordó la falta de estudios biotecnológicos sobre *S. pamplonitana* al desarrollar una metodología para promover la germinación y micropropagación *in vitro*, junto con medidas de control de contaminación para cápsulas de semillas y explantes nodales. Se logró una regeneración exitosa de S. *pamplonitana in vitro* a partir de plantas adultas utilizando segmentos nodales y semillas recolectadas de un único individuo en el invernadero de la Universidad de Pamplona. Los tratamientos de desinfección de la superficie para cápsulas de semillas demostraron el impacto significativo tanto de la concentración de NaOCl como de la duración de la exposición en la contaminación de las semillas y las tasas de germinación. Además, la evaluación de los tratamientos de desinfección de la superficie en segmentos nodales manejó eficazmente los niveles de contaminación y oxidación, resaltando la influencia de la duración de la exposición al fungicida, isodine® y alcohol, con concentraciones más altas y tiempos de exposición más largos que resultaron en una mayor oxidación y una menor contaminación, enfatizando el uso generalizado de hipoclorito de sodio para la esterilización de superficies. La proliferación óptima de brotes se observó con explantes nodales en medio de Murashige y Skoog (MS) suplementado con 2.0 mg/l^{-1} (8.8 µM) de 6-bencilaminopurina y 0.05 mg/l^{-1} (0.14 µM) de ácido giberélico (GA_3), mientras que el enraizamiento fue exitoso en medio MS suplementado con 3.0 mg/l^{-1} (14.7 µM) de ácido indol butírico. Los explantes enraizados se aclimataron a condiciones *ex vitro*. Además, el estudio demostró una germinación mejorada mediante el tratamiento de escarificación y ausencia de luz, ofreciendo beneficios potenciales para la preservación, restauración y reestablecimiento de las poblaciones de *S. pamplonitana* en su hábitat natural.

Palabras clave: *Salvia pamplonitana*; en peligro crítico; regeneración *in vitro*.

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INTRODUCTION

In Colombia, the Labiatae (labiates) family is represented by 23 genera and approximately 203 species and subspecies, of which 183 are native and 17 are endemic. These genera are widely distributed in the peripheries of the high Andean or sub-Andean Forest (*Salvia, Lepechinia, Stachys, Satureja and Obtegomeria)* and moors or submoors *(Stachys, Satureja, Obtegomeria*) (Fernández-Alonso, 2006). Salvia distinguishes itself with around one thousand species (Frodin, 2004), making it the genus with the largest number of species, within the Labiatae family. It is of particular interest due to its diverse essential oils and cosmetic and antitumor qualities (Fennell & Van Staden, 2001) (Kamatou *et al*., 2008; Fernández-Alonso, 1995; Fernández-Alonso & Rivera-Díaz, 2006; Poulios *et al*., 2020).

These plants are characterized by their aromatic properties, comprising mainly herbs or shrubs, with very few trees, and possessing volatile essential oil-secreting glands. Among these, *Salvia pamplonitana* stands out, uniquely known from its type locality in the municipality of Pamplonita, Norte de Santander, and a single collection exclusive to Colombia at the Herbario-Catatumbo-Saráre (Fernández-Alonso & Rivera-Díaz, 2006). Currently, only two plants from this species are cultivated in the greenhouse at the University of Pamplona as the natural populations face threats from habitat loss due to human activities such as urbanization and agricultural expansion. Consequently, the wild populations of *S. pamplonitana* are classified as critically endangered (CR). Given this situation, the study of *Salvia* species in Colombia holds significant interest and relevance, especially in understanding their conservation needs and potential medicinal properties (Fernández-Alonso & Rivera-Díaz, 2006).

Therefore, plant biotechnology emerges as a practical tool for the propagation, restoration, and conservation of ecosystems, as well as the multiplication of biodiversity. In the specific case of *S. pamplonitana*, large-scale *in vitro* propagation is presented as an alternative to reduce the pressure of habitat loss and illegal collection in attempts to satisfy commercial needs. *In vitro* culture techniques of plant species tissues are applied for the rapid, economical, and mass production of plant species valuable for their medicinal and ornamental properties, or those fundamentally in danger of extinction (Shekhawat *et al*., 2015). Despite the significance of such techniques, there is currently no published scientific literature on tissue culture studies for this species at both national and international levels. Therefore, the aim of the current study was to determine an efficient *in vitro* method for the fast propagation of this native and threatened plant species, providing essential support for local conservation plans.

MATERIALS AND METHODS

The research was divided into four experiments as shown in Figure 1: the first and the second consisted of assessing the disinfection protocols for both seed capsules and nodal segments (micro cuttings). The third experiment involved the evaluation of the three stages (establishment, multiplication, and rooting) of micropropagation, while the fourth experiment aimed to improve seed germination.

Plant material

Two types of explants, seed capsules and nodal segments (micro cuttings) of *S. pamplonitana,* were collected from the open greenhouse of the University of Pamplona, Pamplona, Norte de Santander, Colombia (7° 23'12N, 72°58'50W), as shown in Figure 2A.

Disinfection and incubation of plant cultures

The plant material collected was carried in sealed plastic bags to the Tissue Culture Laboratory of the Department of Biology Pamplona, Norte de Santander Colombia. Within the laminar flow chamber, stem segments containing two axillary buds and seed capsules were excised and disinfected.

Disinfection of seed capsules (Experiment I)

The surface of the seed capsules was washed under running tap water for 10–20 minutes and then soaked in a commercial liquid detergent solution (1% v/v) for 10 minutes. Subsequently, the capsules underwent six different disinfection treatments, each with its respective control, followed by five washes with sterile water (Table 1). Finally, the capsules were opened in a laminar flow cabinet, and twenty seeds were extracted. The seeds were then thoroughly washed with sterile distilled water and incubated on a one-tenth (1/10) strength Murashige and Skoog (MS) medium supplemented with 0.7% agar (w/v) Plant TC (PhytoTech Labs®, USA) (referred to as the germination medium) for a period of 6 weeks at 23 ± 2°C under a 16/8 h photoperiod (light/dark) provided by cold white light from 6500 K fluorescent tubes regulated by a timer. The experiment lasted 42 days, with weekly evaluations.

Disinfection and establishment of nodal segments (micro cuttings) (Experiment II)

Stem segments from young branches measuring 25-50 cm in length were collected and transported to the *in vitro* plant culture laboratory at the University of Pamplona. The branches were carefully cut to obtain nodal segments (micro cuttings) ranging from 3-5 cm in length, each containing an axillary bud. The explants were subsequently immersed in commercial liquid detergent (1% v/v; 10 min) to eliminate surface contaminants. They were then subjected to four different surface disinfection treatments, as outlined in Table 2.

Once the superficial disinfection process was completed, the explants underwent five rinses with sterile distilled water to remove any residual disinfectant and were then placed in a 150 mg/L^{-1} citric acid solution to control oxidation during sowing. The disinfected explants were further trimmed to obtain micro cuttings, each with an axillary bud measuring 2 cm in length. Subsequently, each explant was transferred to glass flasks containing supplemented MS Plant Medium (MSPM), (PhytoTech Labs®, USA), establishment medium 30 g L^{-1} sucrose (Merk®, Bogotá, Colombia), 0.1 g L^{-1} myo-inositol

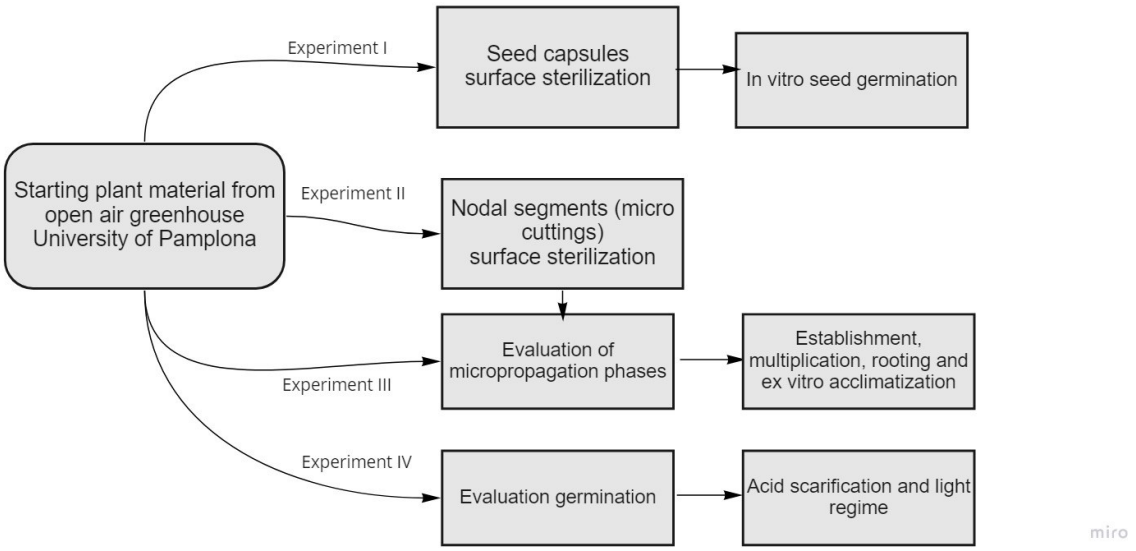

Figure 1. Schematic overview of the different experiments to evaluate *in vitro* propagation of *Salvia pamplonitana*.

Table 1. Surface disinfection treatments evaluated in seed capsules.

	Control	T_1	T_2	T_3	T_4	T_5	T_6
Component							
Isodine® 2%.	0	10 M	10 M	10 M	10 M	10 M	10 M
Fungicide Mertec® 2.5g L^{-1}	0	60 M	60 M	60 M	60 M	60 M	60 M
Alcohol 70%	0	60 S	60 S	60 S	60 S	60 S	60 S
NaOCl 1%	0	60 M					
NaOCl 2%	0		2H				
NaOCl 3%	0			3 H			
NaOCl 4%	0				4 H		
NaOCl 5%	0					5 H	
NaOCl 6%	0						6 H

S: seconds M: minutes; H: hours

(Sigma®), and 0.7% agar (Phytotechnology®) with concentrations of gibberellic acid (GA_3) and 6-benzyl aminopurine (BAP) (Phytotechnology®) specified in Medium M_1, M_2, and M_3 as shown in Table 3.

After two weeks, the meristematic apices that emerged from each micro cutting, free of contamination, were carefully removed and transferred to fresh medium to ensure rapid establishment. The pH of the culture medium was adjusted to 5.7 ± 0.1 using potassium hydroxide (KOH) (Merck®, Bogotá, Colombia) and/or 0.1 N hydrochloric acid (HCl) (Merck®, Bogotá, Colombia) prior to autoclaving (121°C, 1 atm, 20 min). The explants were then incubated at 23 ± 5 °C for 6 weeks under a 16/8 h photoperiod (light/dark) using cold white light from 6500 K fluorescent tubes, regulated by a timer.

Shoot induction and multiplication (Experiment III)
For shoot induction, healthy and contaminant-free explants were separated and transferred to the MS multiplication medium, supplemented with concentrations of (GA_3) and (BAP) (Table 3, M_4, M_5 and M_6 media) and 30% sucrose. After four weeks of growth, the new explants were divided and transferred to fresh multiplication. The percentage of explants with shoots was estimated in each case after one month of cultivation. Explants were replaced every month with fresh medium.

Rooting and *ex vitro* acclimatization
Axillary segments were evaluated in MS medium supplemented with 0.0, 1.0, or 3.0 mg L^{-1} of indole-3-butyric acid (IBA) (Sigma-Aldrich, Darmstadt, Germany) for a duration of 3 weeks (Table 3). The explants with developed roots were subsequently transferred to medium without hormones (MWH), and their growth was monitored weekly. Rooted explants were transplanted into plastic pots filled with a mixture of sand and peat moss (1:1, v/v). All plantlets were watered once with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) and were kept in the green house for a period of 10 days for gradual acclimatization.

Germination experiment (Experiment IV)
After defining the best disinfection treatment for the seed capsules, a fourth experiment was designed to improve the germination rates previously determined in experiment I. This assay combined two experimental variables (treatments): scarification of the seeds (acid scarification) and light regime (incubated at 23 ± 5°C in a 16/8 h light regime and in total darkness). The scarification treatment consisted of soaking the seeds in 70% H_2SO_4 (2.5 min and 5.0 min), followed by thorough washing with sterile distilled water before placing the seeds on germination medium.

Statistical design
All experiments were conducted using a factorial arrangement of treatments in a completely randomized design with four replications (Dytham, 2012). The distribution and homogeneity of variance of the variables were analysed applying the Shapiro-Smirnov and the Levene tests, respectively. To assess the effect of different culture media (M_1 to M_9) a one-way ANOVA was performed when the data were normally distributed. A significance level of $p \leq 0.05$ was used to determine significant differences. Additionally, Tukey's multiple comparison test was conducted to compare the treatments statistically. Data were collected over a period of 10

Table 2. Surface disinfection treatments evaluated in nodal segments (micro cuttings).

	Control	T_1	T_2	T_3	T_4
Component					
Isodine® 2%.	0	5 M	10 M	5 M	10 M
Fungicide Mertec® 2.5g L^{-1}	0	30 M	60 M	30 M	60 M
Alcohol 70%	0	30 S	60 S	30 S	60 S
NaOCl 2%	0	5 M			
NaOCl 2%	0		10 M		
NaOCl 3%				5 M	
NaOCl 3%					10 M

S: Seconds; M: minutes

Table 3. Effect of BAP, GA3 and IBA on the *in vitro* propagation stages of *S. pamplonitana*

	Growth Regulators		
Phases	**6-benzyl amino purine**	**Gibberellic acid**	**Indole butyric acid**
Establishment	**(BAP mg/L)**	**(GA_3 mg/L)**	**(IBA mg/L)**
M_1	0.0	0.0	0.0
M_2	1.0	0.5	0.0
M_3	2.0	1.0	0.0
Multiplication			
M_4	0.0	0.0	0.0
M_5	2.0	0.03	0.0
M_6	2.0	0.05	0.0
Rooting			
M_7	0.0	0.0	0.0
M_8	0.0	0.0	1.0
M_9	0.0	0.0	3.0

weeks and the statistical program SPSS (Statistical Package for the Social Sciences), Version 27 (SPSS, Inc., Chicago, IL) was used to analyse the data.

RESULTS AND DISCUSSION

Effect of disinfection treatments on seed capsules (Experiment I)

Several authors have observed that seeds of wild species collected in the open fields are often severely infected by a diversity of fungi (Halloin, 1975; Klich, 1986; Barampuram *et al.*, 2014) contrasting with those from controlled environments (Niedz & Bausher, 2002). Therefore, germinating heavily contaminated seeds *in vitro* poses a particular challenge, especially when supply is limited. In the case of *S. pamplonitana*, a wild species, previous surface disinfection tests revealed high levels of contamination by fungi and yeasts, most probably attributed by the presence of trichome on the capsule surface that houses the seeds (tetranucula fruit) (Figure 2B), which retains several types of contaminants (Fernández-Alonso & Rivera-Díaz, 2006).

Thus, the study revealed a significant impact of concentration and exposure time to NaOCl on seed contamination and germination rates. Particularly, in T_2 treatment (preceded by imbibition in Vitavax® for 30 minutes and Mertect® for 60 minutes, followed by exposure to 2% NaOCl for 2 hours), contamination rate was reduced to 13.5%, while achieving a germination rate of 71.4% after

Figure 2. A. Specimen *Salvia pamplonitana* greenhouse University of Pamplona; **B.** Seeds in capsule note the high density of trichomes; **C.** Seed germination at 14 days of sowing; **D.** *In vitro* plantlet developed at 28 days of sowing. Bar= 0.5 mm.

28 days of cultivation (Figure 3). These results contrast with those observed in Treatments T_3 to T_6, where increased capsule oxidation adversely affected germination. It is noteworthy that some authors have suggested that sodium hypochlorite is effective in controlling bacteria, fungi, and viruses by oxidizing biological molecules such as proteins and nucleic acids (Çavusoglu *et al*., 2019).

Effect of surface disinfection treatments evaluated in nodal segments (micro cuttings) (Experiment II)

The results also confirmed that treatments T_3 (10.3, 16.3) and T_4 (9.2, 43.2) demonstrated superior effectiveness in controlling both contamination and oxidation levels among the four treatments evaluated for superficial disinfection of stem segments (Figure 4). Additionally, the duration of exposure to the fungicide, isodine®, and alcohol significantly contributed to the successful management of surface disinfection in the micro cuttings. Furthermore, a clear correlation was observed between higher concentrations of the applied compounds and longer exposure times with increased oxidation and reduced contamination. It is well-documented that sodium hypochlorite (NaClO) is widely utilized for surface sterilization of plant tissues in *in vitro* propagation procedures, with concentrations and exposure times tailored to specific species and explant types (see Ding *et al*., 2011; Çavusoglu *et al*., 2019; Barampuram, 2014).

Effect of culture media M_1, M_2 and M_3 on the establishment phase in nodal segments (micro cuttings) of *S pamplonitana* (Experiment III)

The study also unveiled significant differences ($p < 0.05$) in explant development during the establishment phase, with the M_2 medium exhibiting the greatest growth, particularly in meristematic cells within the apexes, thus facilitating better explant development. Moreover, the combination of GA_3 and BAP in micro cutting culture resulted in notably high numbers of leaves (14.3 leaves) and survival rate (95.3%) compared to other treatments (Table 4). While such observations might be novel for *Salvia* species, similar outcomes have been documented in other plant species like *Rubus*, thus corroborating the effectiveness of the identified approaches (Muñoz and Reyes 2006; Sigarroa-Rieche and García-Delgado, 2011).

Effect of culture media M_3, M_4 and M5 on Shoot multiplication phase in nodal segments (micro cuttings) of *S pamplonitana* (Experiment III)

After four weeks of establishing the explants in the multiplication media, significant differences were observed among treatments ($p < 0.05$) for the variable's average growth and number of shoots (refer to Table 5). Specifically, the M_6 medium yielded superior results, generating a greater number of shoots and exhibiting better average growth compared to the M_4 and M_5 media.

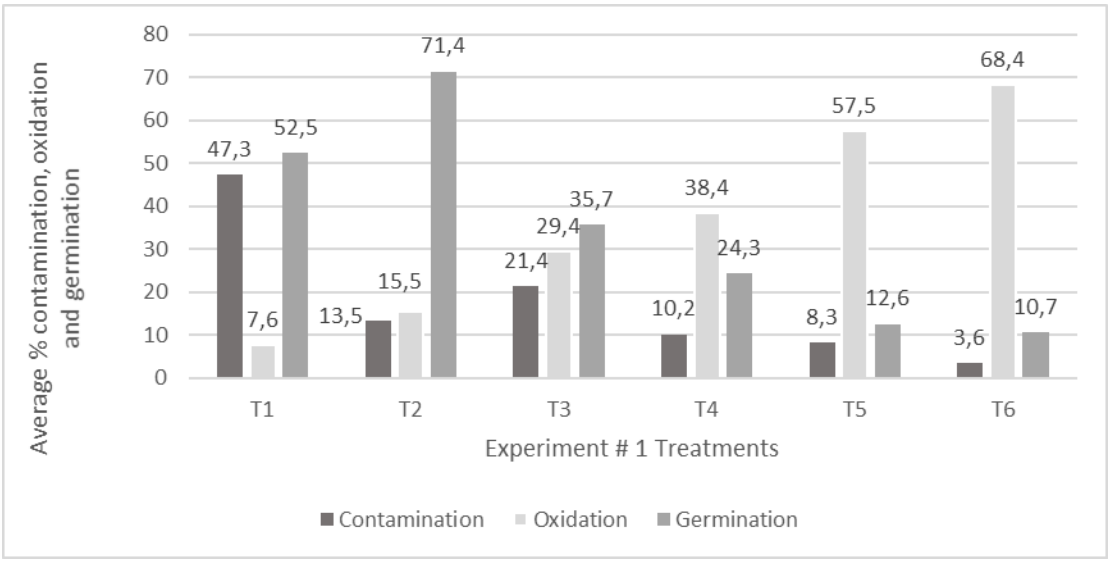

Figure 3. Effect of disinfection treatments on *S pamplonitana* seed capsules.

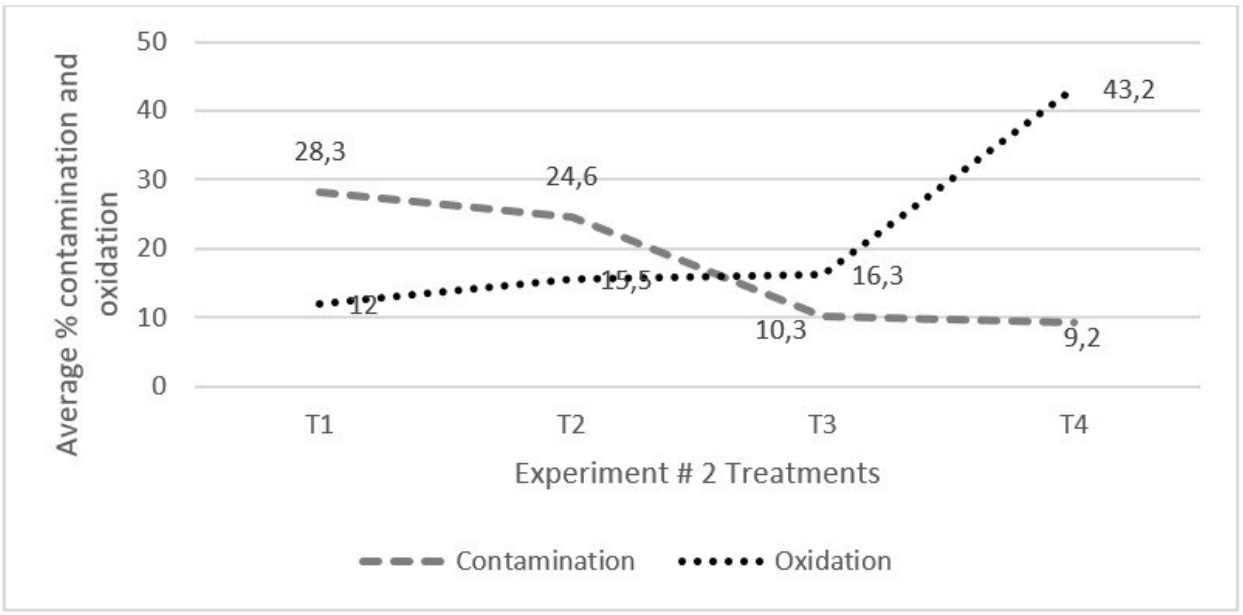

Figure 4. Effect of disinfection treatments on *S. pamplonitana* stem segments

Furthermore, the synergistic effect of BAP combined with GA_3 was observed, promoting the proliferation and elongation of shoots *in vitro*. These results are similar to those described in other species by Vengadesan and Pijut (2009), in explants of *Quercus rubra*, Q. *leucotrichophora* and Muñoz and Reyes (2006), Sigarroa-Rieche and García-Delgado (2011) in *Rubus glaucus*.

Effect of culture media M_7, M_8 and M_9 on *in vitro* rooting phase in nodal segments (micro cuttings) of *S pamplonitana* (Experiment III)

The M_9 medium demonstrated the most favorable results, with nodal segments (micro cuttings) consistently exhibiting a homogeneous average root number ranging from 4.6 to 2.8 roots per plant after four weeks of cultivation across treatments (Table 6 and Figure 2 C, D). During this phase, both endogenous and exogenously supplemented auxin (IBA) favored the development of a robust root system suitable for transplanting plant material of *S. pamplonitana* and its subsequent establishment in *ex vitro* conditions. This effect aligns with the findings of George et al. (2008), who highlighted the significant influence of auxins on micro cuttings, aiding in the organization of meristems for root formation, cell growth, expansion, and vascular tissue differentiation, among other processes. It is important to emphasize that the percentage of survival in the three treatments did not exhibit significant differences.

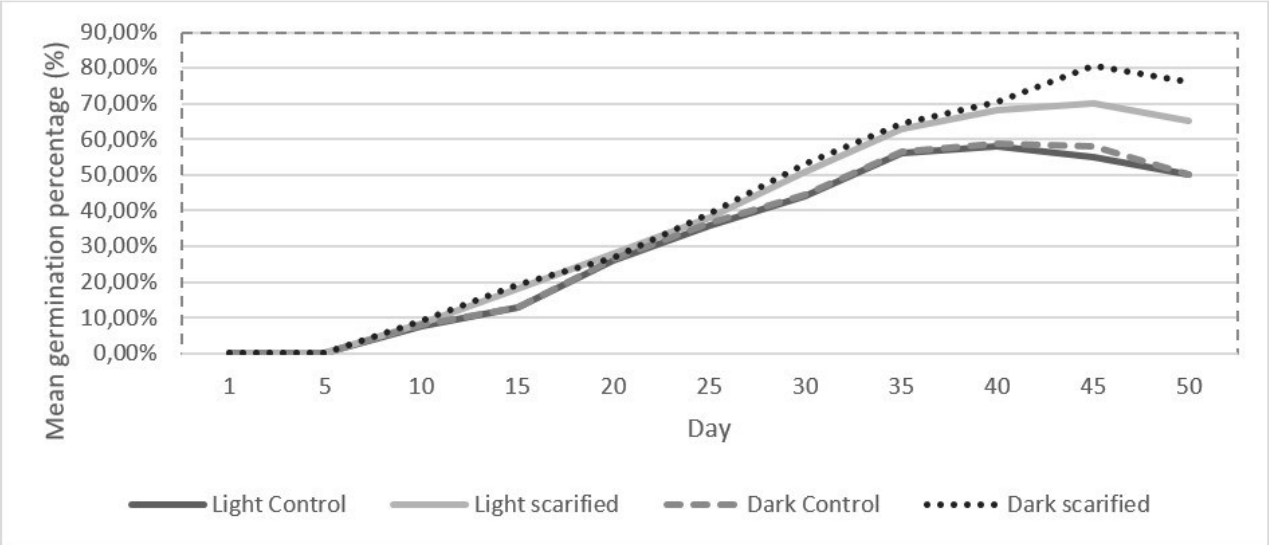

Figure 5. The germination frequency (%) of *S. pamplonitana* seeds was monitored under two light conditions over a period of fifty days, with additional treatment involving chemical scarification.

Table 4. Tukey's multiple comparison test on the effect of culture media on the establishment phase in nodal segments (micro cuttings) of *S. pamplonitana*.

Treatment	No of leaves		Survival percentage
M1	7,789	c	78,7
M_2	14,367	B	95,3
M3	22,675	A	82,2

Figures followed by different letters are significantly different at $p < 0.05$, using Tukey's multiple range test.

Table 5. Tukey's multiple comparison test of the effect of the culture media on the average growth and the number of shoots in the multiplication phase of *S pamplonitana*.

Treatment	Average growth		Number of shoots		Survival percentage
M_4	3,188	b	2,578	b	69,3
M_5	5,156	b	4,513	b	74.3
M_6	7,075	a	6,800	a	83,5

Figures followed by dissimilar letters are significantly different at $P<0.05$, using Tukey's multiple range test.

Germination experiment (Experiment IV)

To improve germination, *S. pamplonitana* seeds were exposed to either light or chemical scarification and as reported in other *Salvia* species (Kamatou *et al*; 2008, Kamatou and Viljoen 2010), the germination of *S. pamplonitana* seeds exhibited a sigmoid pattern over 49 days (Figure 5). As a general tendency, the first signs of germination were noted in seeds grown under both dark and light conditions seven days after culturing. However, germination rates slowed down after day forty and declined further from day fifty. Statistical analysis (ANOVA) showed that the type of treatment significantly influenced the day in which germination initiation occurred. Specifically, germination rates were higher in seeds subjected to dark conditions and chemical scarification, and lower in seeds exposed to light without scarification (Figure 5). These findings are consistent with those described by Hannibal *et al*. (2010), who highlighted that *in vitro* germination of *S. stenophylla* is favored by chemical scarification.

CONCLUSION

This is the first description on *in vitro* propagation using nodal segments in *S. pamplonitana*, a critically endangered plant species. Through scarification treatment and light deprivation, significant improvements in germination rates were achieved, highlighting the potential of biotechnological tools in species preservation. These findings underscore the importance of advancing scientific understanding to address conservation challenges effectively. By providing effective propagation methods, this research contributes to bolstering local conservation efforts and safeguarding the future of *S. pamplonitana* populations.

CONFLICT OF INTEREST

The manuscript was prepared and reviewed with the participation of all the authors, the authors declare that there is no conflict of interest in this study.

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