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In vitro establishment of the phytoremediator species totora (*Schoenoplectus californicus*) from meristematic tissues

Establecimiento *in vitro* de la especie fitorremediadora totora (*Schoenoplectus californicus*) a partir de tejidos meristemáticos

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Article Data

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Abstract

Schoenoplectus californicus (totora) is an aquatic macrophyte of high ecological relevance and potential application in phytoremediation. However, its sexual propagation is limited due to the low germination percentage and reduced seed viability, which hinders the production of homogeneous plant material. In this context, the objective of the present study was to establish the species in vitro from meristematic corms, aiming to obtain plant material suitable for subsequent micropropagation. During the disinfection phase, different concentrations of sodium hypochlorite and immersion times were evaluated, while in the establishment phase, various agar concentrations were tested in MS medium supplemented with growth regulators. The variables assessed included contamination, oxidation, and meristem survival. Results indicated that disinfection treatments significantly influenced contamination and oxidation of explants. Likewise, lower agar concentrations favored meristem survival, with the highest average obtained at 1 g of agar. It is concluded that the concentration of the gelling agent is a determining factor for the in vitro establishment of *S. californicus*. Low agar concentrations proved to be the most suitable for promoting explant survival and ensuring the availability of viable plant material. These findings provide relevant information for future micropropagation and phytoremediation studies, contributing to the sustainable use and conservation of this species in Andean aquatic ecosystems

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Resumen

Schoenoplectus californicus (totora) es una macrófita acuática de gran relevancia ecológica y con potencial aplicación en procesos de fitorremediación. No obstante, su propagación sexual presenta limitaciones debido al bajo porcentaje de germinación y la reducida viabilidad de sus semillas, lo que dificulta la obtención de material vegetal homogéneo. En este contexto, el objetivo de la presente investigación fue establecer *in vitro* la especie a partir de cormos meristemáticos, con el propósito de disponer de material vegetal apto para la fase de micropropagación. Durante la fase de desinfección se evaluaron diferentes concentraciones de hipoclorito de sodio y tiempos de inmersión, mientras que en la fase de establecimiento se analizaron distintas concentraciones de agar en medio de cultivo MS suplementado con reguladores de crecimiento. Las variables consideradas fueron contaminación, oxidación y sobrevivencia de los meristemos. Los resultados expusieron que los tratamientos de desinfección influyeron significativamente en la contaminación y oxidación de los explantes. Asimismo, se evidenció que las menores concentraciones de agar favorecieron la sobrevivencia, alcanzando el mayor promedio con 1 g de agar. Se concluye que la concentración del agente gelificante constituye un factor determinante para el establecimiento in vitro de *S. californicus*. Las bajas concentraciones de agar resultan más apropiadas para promover la sobrevivencia de los explantes y garantizar la obtención de material vegetal viable. Estos hallazgos aportan información relevante para futuros estudios de micropropagación y fitorremediación, contribuyendo al aprovechamiento sostenible y la conservación de esta especie en sistemas acuáticos andinos

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Introduction

In Bolivia, the most relevant advances in plant biotechnology were mainly oriented towards the massive propagation of agricultural species of economic interest[1]; however, there is still limited research on the propagation and conservation of native species with ecological potential and phytoremediation capacity[2]. Among these species, *Schoenoplectus californicus* (totora) stands out, an aquatic macrophyte widely distributed in the circumlacustrine area of Lake Titicaca, with adaptation and growth in humid environments and muddy soils.

The totora reed plays a fundamental ecological role in the high Andean lake ecosystems, by intervening in water regulation, the protection of aquatic habitats and the recycling of nutrients. However, Lake Titicaca has suffered progressive environmental degradation, associated with both anthropic and natural factors, including the contamination of tributaries, accumulation of heavy metals, deterioration of water quality, loss of biodiversity and alterations in aquatic ecosystems[3]. Added to this are the effects of climate change, which modify environmental conditions and affect the distribution of aquatic macrophytes.

In this context, *S. californicus* was identified as a species with high phytoremediating potential, thanks to its ability to absorb and remove pollutants present in water bodies. Research has reported its efficiency in the capture of heavy metals and in the reduction of compounds such as nitrogen, phosphorus and coliform bacteria[4-6]. Likewise, its capacity to capture carbon dioxide has been highlighted, which reinforces its ecological importance in environmental restoration programs.

However, the sexual propagation of this species has limitations related to the low percentage of germination, the presence of dormancy and the probable hybrid origin of some populations, factors that reduce the viability of seeds and make it difficult to obtain uniform plant material[7-10]. In addition, sexual reproduction generates high genetic variability, while vegetative propagation allows the conservation of characteristics of ecological and physiological interest[11,12].

Faced with this problem, plant tissue culture is an efficient biotechnological alternative for the propagation and conservation of *S. californicus*. *In vitro* establishment allows genetically homogeneous plant material to be obtained under controlled conditions, in addition to being a useful tool for future research in micropropagation, conservation, and phytoremediation processes[13].

Therefore, the objective of the present research was to establish *in vitro* the phytoremediator species *S. californicus* from meristematic corms, in order to have plant material suitable for the micropropagation phase.

Materials and methods

Plant material. The collection of the plant material was carried out in April 2023 in the port of Guaqui, Ingavi province of the department of La Paz, Bolivia, located on the shores of Lake Titicaca at 3811 meters above sea level. Plants with actively growing shoots and morphological characteristics were selected. Subsequently, 600 vegetative shoots were collected for the *in vitro* establishment.

Disinfection of explants. The explants were washed with running water to remove sediment and organic matter. They were then initially immersed in 70 % alcohol for 1 min. Disinfection was carried out by

means of sodium hypochlorite treatments at concentrations of 2, 3 and 4 % during immersion times of 5, 10 and 15 min, adapting protocols previously used in *in vitro* culture of plant species[14].



Figure 1 Collection of plant material in the port of Guaqui in the municipality of Guaqui in the department of La Paz

Table 1 Design of disinfection treatments

Factor A	Factor B	Treatment	Replays		
NaClO (%)	T inm (min)	N°	I	II	III
	5	T_1	T_1	T_1	T_1
2	10	T_2	T_2	T_2	T_2
	15	T_3	T_3	T_3	T_3
	5	T_4	T_4	T_4	T_4
3	10	T_5	T_5	T_5	T_5
	15	T_6	T_6	T_6	T_6
	5	T_7	T_7	T_7	T_7
4	10	T_8	T_8	T_8	T_8
	15	T_9	T_9	T_9	T_9
Total	540 tubos				

Variable A: Sodium hypochlorite; Variable B: Immersion time, (NaClO) Sodium hypochlorite, (T inm) immersion time

Table 2. Treatment design

Treatment	Component
T_1	M&S (100%) + 10 mL de BAP + 1 g. of agar
T_2	M&S (100%) + 10 mL de BAP + 3 g. of agar
T_3	M&S (100%) + 10 mL de BAP + 5 g. of agar
T_4	M&S (100%) + 10 mL de BAP + 2mL de IBA 1 g. of agar
T_5	M&S (100%) + 10 mL de BAP + 2mL de IBA 3 g. of agar
T_6	M&S (100%) + 10 mL de BAP + 2mL de IBA 5 g. of agar

After disinfection, the explants were rinsed three times with sterile distilled water under aseptic conditions.

Culture medium. The basal medium Murashige & Skoog (MS)[15] supplemented with 30 g L^{-1} sucrose,

0.1 g L^{-1} myo-inositol and BAP and IBA growth regulators were used according to the corresponding treatment. The pH was adjusted to 5.7 by 1N NaOH. Agar concentrations of 1, 3 and 5 g L^{-1} were evaluated as a gelling agent. The medium was then distributed in test tubes and autoclaved at 121° C for 15 min.

In vitro establishment. The meristems were dissected and established in the different culture media under aseptic conditions. The explants were incubated at a temperature of 25 ± 1° C, relative humidity of 70-80 % and with a photoperiod of 14 h.

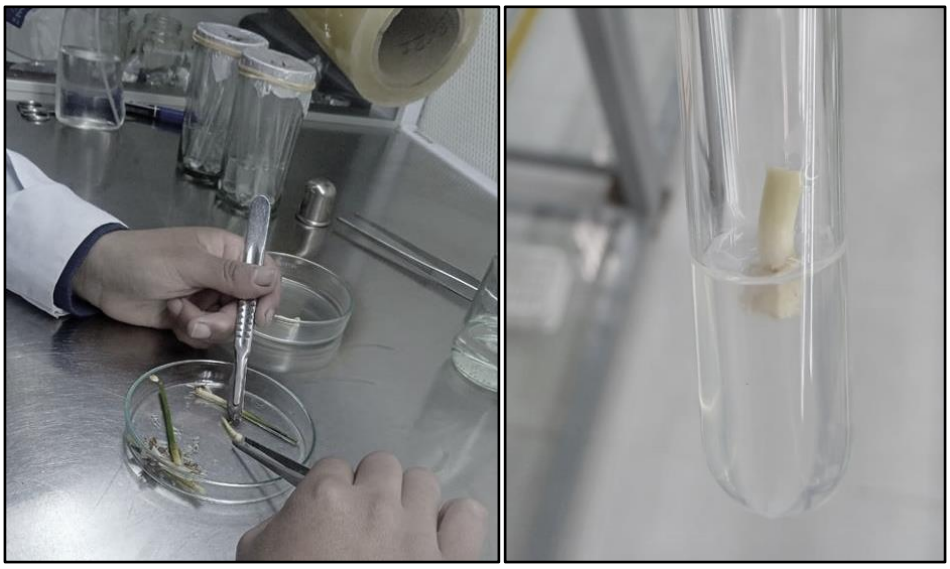


Figure 2 In *vitro establishment*. A: Establishment of the totora; B: Meristem in the culture medium with 3 grams of agar

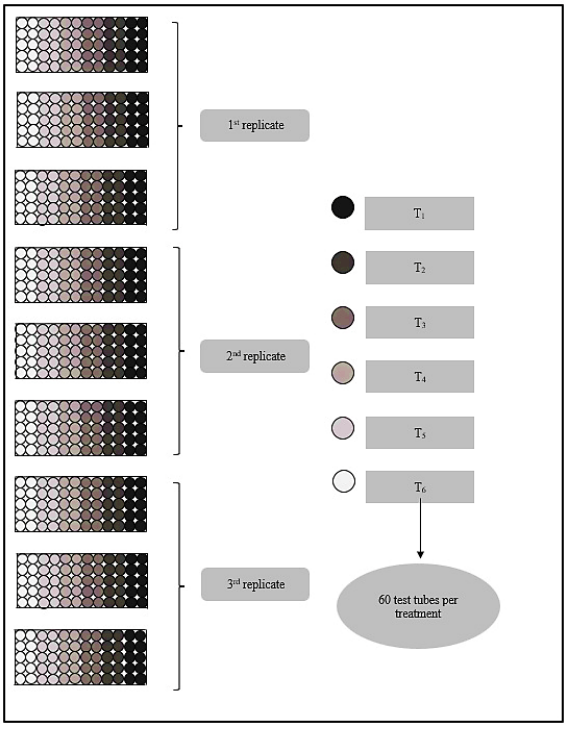


Figure 3 Sketch of the experimental design

Evaluation. At 20 days after planting, 60 explants were evaluated for each treatment. The variables analyzed were; percentage of contamination, oxidation and survival (explants that managed to establish themselves and did not become contaminated or oxidized)

Treatments, experimental design and statistical analysis. In the disinfection phase, 9 treatments were evaluated using a completely randomized design with a two-factor arrangement (to determine the influence of the different concentrations of sodium hypochlorite and immersion time on the *in vitro* establishment of *S. californicus* explants) considering sodium hypochlorite concentration as factor A and immersion time as factor B.

For the establishment phase, 6 treatments were evaluated under a completely randomized design with a two-factor arrangement (to evaluate the effect of

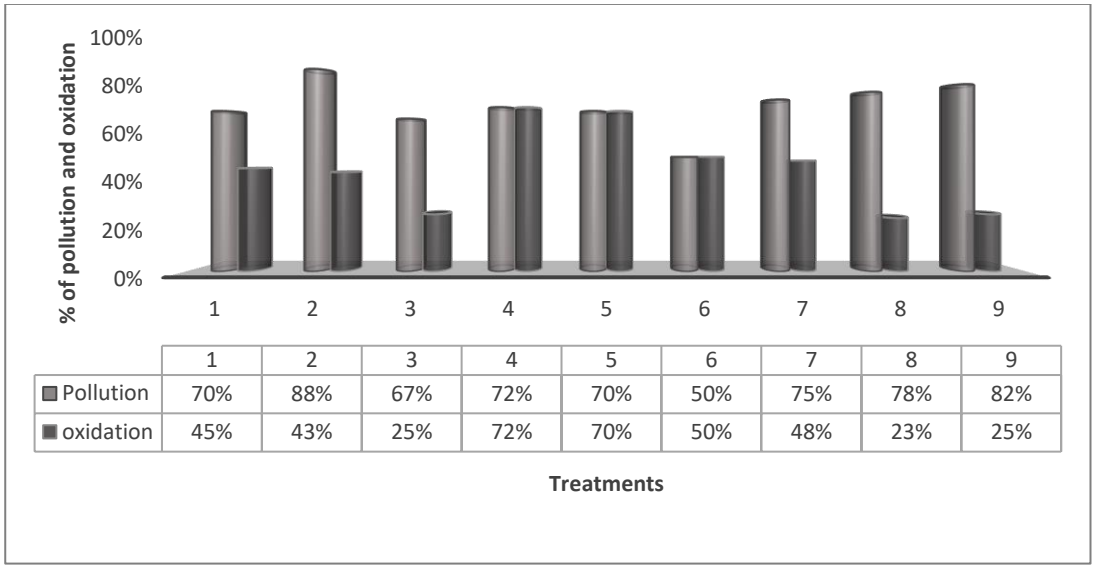
agar in vitro plants) considering the type of culture medium as factor A and factor B the agar concentration. Each treatment had three repetitions.

The data were analyzed by analysis of variance (ANOVA) using the InfoStat program. Significant differences between treatments were also determined using Duncan's multiple comparison test.

Results

Results of the disinfection of meristematic corms. The ANOVA showed no significant differences between treatments for the percentage of contamination variable ($p > 0.05$). However, variations in the behavior of the explants were observed during the *in vitro* establishment phase.

Figure 4 shows the percentages of contamination and oxidation recorded in the different treatments evaluated. Treatment 6 had the lowest percentage of contamination (43 %), while treatment 8 registered the lowest percentage of oxidation (13 %).



	1	2	3	4	5	6	7	8	9
Pollution	70%	88%	67%	72%	70%	50%	75%	78%	82%
oxidation	45%	43%	25%	72%	70%	50%	48%	23%	25%

Figure 4 Percentage of contamination and oxidation by treatment

In general, treatments with higher concentrations of sodium hypochlorite and longer immersion times tended to reduce contamination levels; however, some treatments increased oxidation of meristematic tissues.

Duncan's multiple comparison test showed significant differences for the oxidation variable, with higher percentages observed in treatments with 3 % sodium hypochlorite and prolonged immersion times.

During the establishment phase, the explants presented morphological changes characterized by initial growth and variations in tissue coloration, with different levels of adaptation observed *in vitro* depending on the treatment applied.

Results of the effect of agar on the establishment in vitro. Figure 5, the survival percentage of meristems in the different treatments evaluated. Treatments with 1 g of agar had the highest survival percentages, while high agar concentrations registered lower values.

The analysis of variance showed highly significant differences between agar concentrations for the survival variable ($p < 0.01$). Treatments with lower agar concentration had higher survival percentages compared to media with higher levels of gelation.

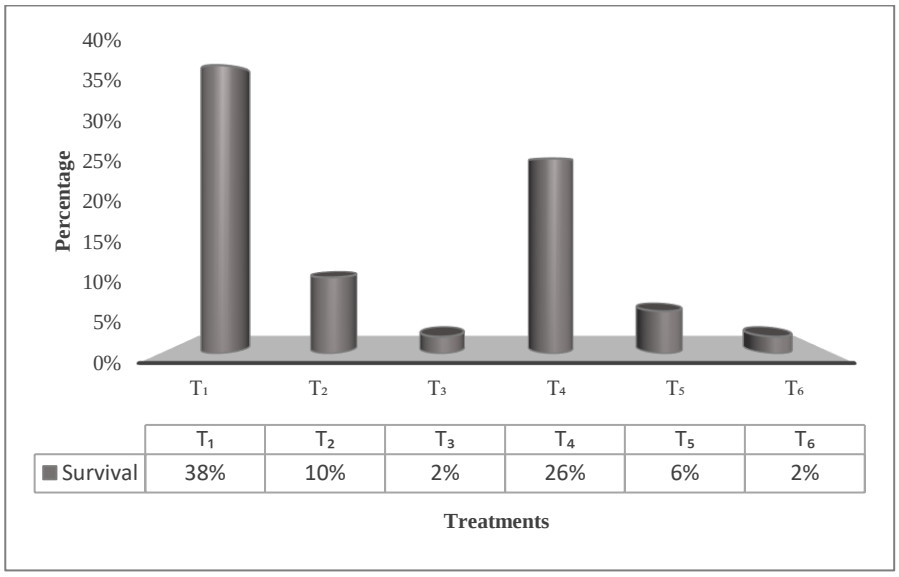


Figure 5 Survival in the different treatments

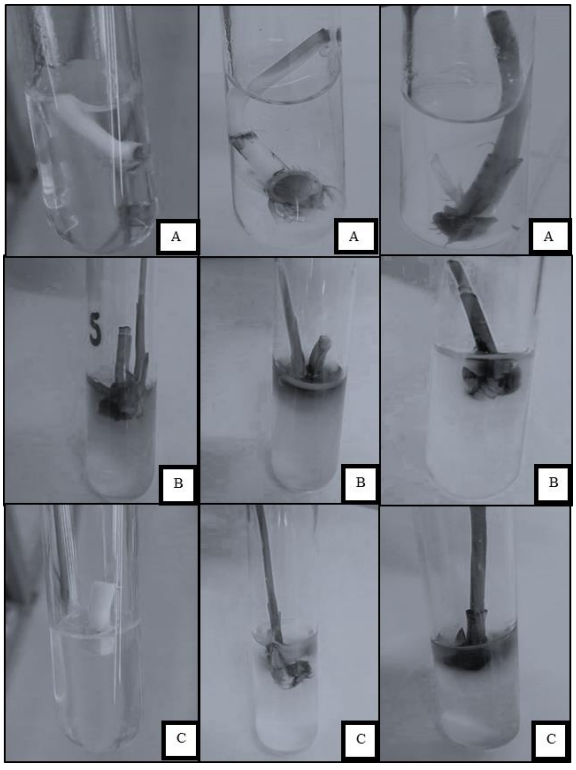


Figure 6 Growth of the reed. A: Growing in culture medium with 1 g of agar; B: Growing in culture medium with 3 g of agar; C: Growing in culture medium with 5 g of agar

Duncan's test indicated that treatment with 1 g L^{-1} agar achieved the highest average survival rate (34.06 %), statistically differentiating itself from treatments with 3 and 5 g L^{-1} agar.

It was also observed that high concentrations of agar reduced the growth and viability of the explants during the initial phase of *in vitro* establishment.

In general terms, the media with a lower concentration of gelling agent favored the establishment of the meristems and allowed a greater survival of the vitro plants.

Discussion

The results obtained in the present study showed that the *in vitro* establishment of *S. californicus* was mainly conditioned by two factors: the efficacy of the disinfection process and the concentration of the gelling agent used in the culture medium. Both factors directly influenced the viability, survival and physiological response of meristems during the early stages of cultivation.

Table Analysis of variance for the variable: Survival

F.V	G.L	S.C	C.M	F	p-valor
Culture medium	1	90.36	90.36	1.70	0.2172 NS
Agar Agar Levels	2	2525.45	1262.73	23.71	0.0001 **
M*NA	2	81.61	40.81	0.77	0.4863 NS
Error	12	639.16	53.26		
Total	**17**	**3336.58**			

Table 4 Duncan Test for Agar Amounts

Agar levels	Average or Averages	Duncan
1	34.06	A
3	13.98	B
5	5.88	B

During the disinfection phase, differences were observed in the levels of contamination, oxidation and survival of the explants. Although high concentrations of sodium hypochlorite contributed to partially reduce microbial contamination, they also increased oxidative damage in meristematic tissues. This behavior suggests that there is a critical balance between disinfectant efficiency and preservation of cell viability during *in vitro* establishment.

The observed oxidation was probably associated with the release of phenolic compounds caused by cell damage during the handling and disinfection of the explants. This phenomenon represents one of the main limitations in plant tissue culture, because the accumulation of oxidized compounds affects the regenerative capacity and survival of tissues cultured *in vitro*.

Likewise, the sensitivity of meristems to disinfectant agents can vary according to the physiological state of the explant and the characteristics of each plant species. Once the initial disinfection phase was over, the response of the meristems was significantly influenced by the physical conditions of the culture medium, particularly by the concentration of agar.

Treatments with lower concentrations of gelling agent had higher survival percentages, indicating that the water availability of the medium is a determining factor for the *in vitro* establishment of *S. californicus*. Agar plays a fundamental role in plant tissue culture, as it provErrorides physical support to the explants and regulates the availability of water and nutrients in the culture medium[15,16]. Debergh[17] and Nery et al.[18] pointed out that the physicochemical properties of the gelling agent modify the water potential and mobility of solutes, directly affecting the growth and development of tissues cultured *in vitro*. Similarly, Ghashghaie et al.[19] reported that high concentrations of agar reduce the availability of water for explants, limiting their growth and viability.

The results obtained coincide with these reports, since treatments with higher concentrations of agar presented higher mortality rates. This response could be related to a lower absorption of water, nutrients and growth regulators by meristematic tissues in more solid media. In contrast, several authors indicated that liquid media or media with low concentrations of agar favor nutrient absorption, cytokinin availability, and system aeration, promoting better development of *in vitro* cultured explants[20-22]. These conditions could explain the better performance observed in treatments with lower agar concentration during the establishment stage.

However, although the low concentrations of agar favored the initial survival of the meristems, during the multiplication phase an increase in the symptoms of hyperhydration and mortality was observed. This

phenomenon, also called vitrification, is a common physiological alteration in plant tissue culture and is associated with an excess of water available in *in vitro* cultured tissues[23].

Hyperhydration negatively affects the anatomy and physiology of vitro plants, causing alterations such as cuticle reduction, chloroplast disorganization and decreased photosynthetic efficiency[24,25]. Likewise, Hazarika[26] pointed out that this disorder significantly compromises the quality and acclimatization capacity of the vitro plants due to structural and physiological alterations of the tissues. Among the main factors associated with this phenomenon are low agar concentrations and inadequate osmotic potentials[26,27].

The results of the present study suggest that, although less gelled media favor the initial establishment of *S. californicus*, it is necessary to progressively adjust the concentration of the gelling agent as the stages of *in vitro* culture progress, in order to reduce physiological alterations and improve the quality of the propagated material. Similar results were reported by Ebrahim[28] in *Zantedeschia aethiopica* and by Muthukrishnan et al.[29] and Suthar et al.[30] In *Ceropegia thwaitesii* and *Boswellia serrata*, respectively, the response of the explants was closely related to the physical consistency of the culture medium.

Overall, the results obtained indicate that both the disinfection protocol and the agar concentration are critical factors during the *in vitro* establishment of *S. californicus*. The observed response could be related to the aquatic nature of the species, which is more sensitive to variations in the water availability of the environment. These findings represent an important basis for future research aimed at optimizing micropropagation protocols, conservation, and phytoremediation applications in ecologically important aquatic species.

Finally, the use of 70 % alcohol for 1 min, followed by 3 % sodium hypochlorite for 15 min, allowed the *in vitro* establishment of meristematic explants of *S. californicus* with low levels of contamination, constituting the most efficient treatment for *in vitro* establishment of the explants. Likewise, it was pointed out that the concentration of the gelling agent significantly influenced the survival of meristems, obtaining the highest survival percentage (34.06 %) in the culture medium supplemented with 1 g L^{-1} of agar.

These results allowed to establish the most favorable conditions for the *in vitro* establishment of *S. californicus*, fulfilling the objective set in the research and providing a basis for future studies of micropropagation, conservation and phytoremediation of the species.

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Conflicts of interest

There is no conflict of interest.

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technical collaboration during the development of the research.

Ethical considerations

This research was carried out in compliance with the ethical and biosafety standards established by the Military School of Engineering (EMI). The plant material used corresponds to the native species *Schoenoplectus californicus* (totora), collected without affecting natural populations or ecosystems, for exclusively scientific purposes.

Limitations in research

Reduced availability of contamination-free plant material, the time for the establishment of *in vitro* culture, Likewise, the results obtained correspond to a first experimental phase and require additional validations in subsequent phases of micropropagation and field**.**

Authors' contribution

Gladys Limachi Quispe, experimental execution, data recording and initial writing of the manuscript. *Jheanete Pérez Guzmán*, Plant Biotechnology (EMI) laboratory, experimental execution, methodological design, technical supervision, and manuscript correction.

Data Access

The data generated and analyzed during this research are available at the EMI Plant Biotechnology Laboratory.

Consent to publication

The authors give their consent for publication. Likewise, the Military School of Engineering (EMI) authorizes the dissemination of the results obtained within the framework of its research projects.

Use of Artificial Intelligence

No Artificial Intelligence (AI) or generative writing assistance tools were used to create or edit the scientific content, figures, or conclusions of this manuscript.

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