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***Cinchona officinalis* L. in vitro conservation by means of slow-growth storage and cryopreservation techniques**

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Abstract: *Cinchona officinalis* has experienced anthropogenic pressures for nearly 400 years, such as overexploitation, habitat fragmentation, and the subsequent reduction of genetic diversity. Additionally, the challenge of regeneration in its natural environment makes it a vulnerable species. In this context, various treatments for the in vitro conservation of explants were evaluated in the present study. For short-term conservation, the effects of osmotic substances such as sorbitol, mannitol, and sucrose at different concentrations were assessed. Different concentrations of MS and B5 culture media were also examined for their impact on the growth, budding, mortality, and rooting of explants over 12 months without subcultures. For long-term conservation by cryopreservation, two techniques were tested: vitrification and encapsulation-dehydration. Short-term preservation of explants in sorbitol resulted in low mortality, minimal growth, and limited development of new shoots compared to preservation in sucrose or mannitol, although tissues could be recovered successfully from all storage conditions. After cryopreservation and 45 days of recovery, explants with the lowest mortality (4%) were from the control treatment, cultivated in a medium with sucrose which proved useful as a cryoprotectant, followed by the encapsulation-dehydration treatment. In conclusion, it is possible to conserve *C. officinalis* tissues in the short-term using in vitro techniques, while further assays are needed for long-term conservation.

Keywords: *In vitro* conservation; sorbitol; sucrose; encapsulation-dehydration

Introduction

Cinchona officinalis L. (Rubiaceae) is a species native to South America, historically renowned for its medicinal properties in treating malaria (Madsen 2002; Garmendia 2005). According to records, in the province of Loja, *Cinchona* trees were widely distributed. However, historical pressures such as overharvesting and habitat fragmentation (Tapia-Armijos et al. 2015; Arbizu et al. 2021) have led to a reduction in genetic diversity in small populations (Rivera et al. 2008; Cueva-Agila et al. 2019), resulting in the current classification of the species as vulnerable or threatened (Krishnan et al. 2011; Coelho et al. 2020).

In various studies, it has been observed that forest fragmentation impacts the regeneration of populations and leads to a decrease in genetic diversity due to alterations in habitat conditions. Although specific studies on the relationship between habitat fragmentation and regeneration in *C. officinalis* have not been conducted, there is evidence pointing to the species' low capacity for regeneration and natural germination (Sánchez-Santillan et al. 2021; Fernandez-Zarate et al. 2022a; Fernandez-Zarate et al. 2022b). Additionally, factors such as the morphology of *C. officinalis* seeds, characterized by their small size and limited reserves for the survival of germinated seedlings, and the size of the embryo, affecting germination time and rate under natural conditions, also influence this process (Romero-Saritama 2015). Consequently, fragmented populations of *C. officinalis* have been reduced to small patches, with populations on private properties having conservation possibilities, while those subjected to high intervention face the imminent risk of disappearance (Cueva-Agila et al. 2019). Therefore, the conservation of this species requires an integrated strategy that combines both in situ and ex situ conservation approaches (Armijos-González et al. 2021; Coelho et al. 2020). Solely relying on in situ conservation may not be adequate for preserving threatened species (Reed et al. 2011; Pence et al. 2022), especially in cases like that of *C. officinalis* which faces challenges in natural regeneration (Romero-Saritama 2015; Armijos-González and Pérez-Ruiz 2016) and may not always be suitable or feasible due to the nature or threatened status of the species (Walters and Pence, 2020).

Ex situ conservation strategies aim to preserve genetic diversity by maintaining plant specimens outside their natural habitat. These approaches encompass the use of DNA banks, cryopreservation, and in vitro tissue culture (Paunescu 2009; Radhamani and Sivaraj, 2013; Custódio et al. 2022). While seed storage is the most common method for ex situ germplasm conservation, it may not be suitable for certain genotypes (Reed et al. 2011; Cruz et al. 2013; Rajasekharan and Sahijram 2015). This is particularly relevant for species like *C. officinalis*, characterized by high seed production throughout the year, yet with low percentages of mature and viable seeds (Romero-Saritama 2015). Additionally, its relative, *C. pubescens*, undergoes a loss of germinative vigor over time and under storage conditions (Cué et al. 2020). So, a viable alternative to conserve this species, which faces challenges in its natural regeneration and has limited possibilities of preservation through seeds, is tissue conservation (Hunter 1986; Cruz et al. 2013). This method requires a small amount of plant material (Reed et al. 2013), involves low economic costs (Paunescu 2009; Reed et al. 2013), and is based on the principle of regeneration from organized or unorganized tissues, such as seeds, pollen, buds, meristems, tissues, cells, somatic embryos, calluses, and others (Paunescu 2009; Engelmann 2011; Reed et al. 2011).

The conservation of tissues through in vitro cultivation techniques has solidified itself as a valuable alternative, deploying two fundamental approaches: slow-growth storage (achieved by adjusting conditions that directly influence the development of explants) and reducing the frequency of required subcultures (Cruz et al. 2013; Rajasekharan and Sahijram 2015). This method allows extending the duration of a subculture cycle from a few

weeks to 12 months or more (Gianní and Sottile 2015; Oliveira and Ibrahim 2022; Panis et al. 2020; Radhamani and Sivaraj 2013; Li et al. 2014). On the other hand, tissue cryopreservation involves the suspension of growth for indefinite periods (Paunescu 2009; Ai et al. 2012; Reed et al. 2011; Sen-Rong and Ming-Hua 2012; Cruz et al. 2013; Panis et al. 2020; Popova et al. 2023). The application of these techniques translates into a significant contribution to the conservation of species facing challenges in natural propagation (Ruta et al. 2020), safeguarding the genetic diversity of the species in question (Mohanty et al. 2013; San José et al. 2014; Armijos-González et al. 2021).

The evaluation of *in vitro* conservation techniques in *C. officinalis* can support the implementation of effective *ex situ* conservation strategies for the species. In this study, the effectiveness of modifying cultivation conditions was assessed, including the culture medium and the addition of osmotic substances to limit growth. Additionally, pre-treatment approaches for long-term preservation of explants in liquid nitrogen were examined.

Materials and Methods

Plant material

The plant material of *C. officinalis* used in the slow-growth and cryopreservation assays was obtained from 4-month-old seedlings that germinated *in vitro* (Fig. 6A). The seeds were collected from trees growing naturally in the Aguarango sector (Vilcabamba-Loja), in the buffer zone of Podocarpus National Park. The protocol described by Armijos-González and Pérez-Ruiz (2016) was applied for the disinfection and stimulation of germination under *in vitro* conditions.

In vitro conservation

- Slow-growth storage

To reduce growth and ensure the viability of explants for 12 months without the need for subculturing, adjustments were made to the culture medium conditions. In the Gamborg B5 medium, three different osmotic substances were introduced: sucrose, mannitol, and sorbitol, each at three varied concentrations (2%, 4%, and 8%) (Table 1). Additionally, concentrations of the culture media were evaluated, both in the Murashige and Skoog (MS) medium and the Gamborg (B5) medium, each at three different concentration levels (25%, 50%, and 100%) (Table 1). In total, 15 media were analyzed, and the control treatment consisted of the B5 medium at 100% concentration. All culture media were solidified with 0.7% agar and the media pH was adjusted to 5.8 ± 0.02 . All media were autoclaved at 121°C for 20 min (110,000 Pa).

Table 1 Percentages of osmotic substances and culture media

Treatments	Components modified	Percentage (%)
1		2
2	Osmotic substances (in a B5 medium)	4
3		8
4		2
5	Sucrose	4
6		8

7		2
8	Sorbitol	4
9		8
10		25
11	Murashige and Skoog (MS)	50
12		100
13	Culture medium	25
14	Gamborg (B5)	50
15 (control)		100

In all treatments, nodal segments measuring 0.7 to 2 cm with 2 to 5 axillary and apical buds from in vitro germinated seedlings were utilized (Fig. 6A). Five nodal segments were placed in each treatment with five repetitions (culture flasks), and the experiment was repeated three times, totaling 1125 explants analyzed. The explants were cultivated under a 12/12 h photoperiod (day/night) with a photon flux density of $57 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent lamps. The average temperature was maintained at $23 \pm 2^\circ\text{C}$, with a relative humidity of $65 \pm 7\%$.

The evaluation of the response to different treatments involved measurements of explant growth (cm), the number of shoots, the presence/absence of roots, and the condition of the explants (alive/dead). The plant's condition was determined based on coloration, using the Munsell Table from 1998. Shades from green to red were considered as living tissue, while brown and dark brown tones were classified as dead tissue. These assessments were conducted at 0, 3, 6, 9, and 12 months of cultivation.

- Cryopreservation

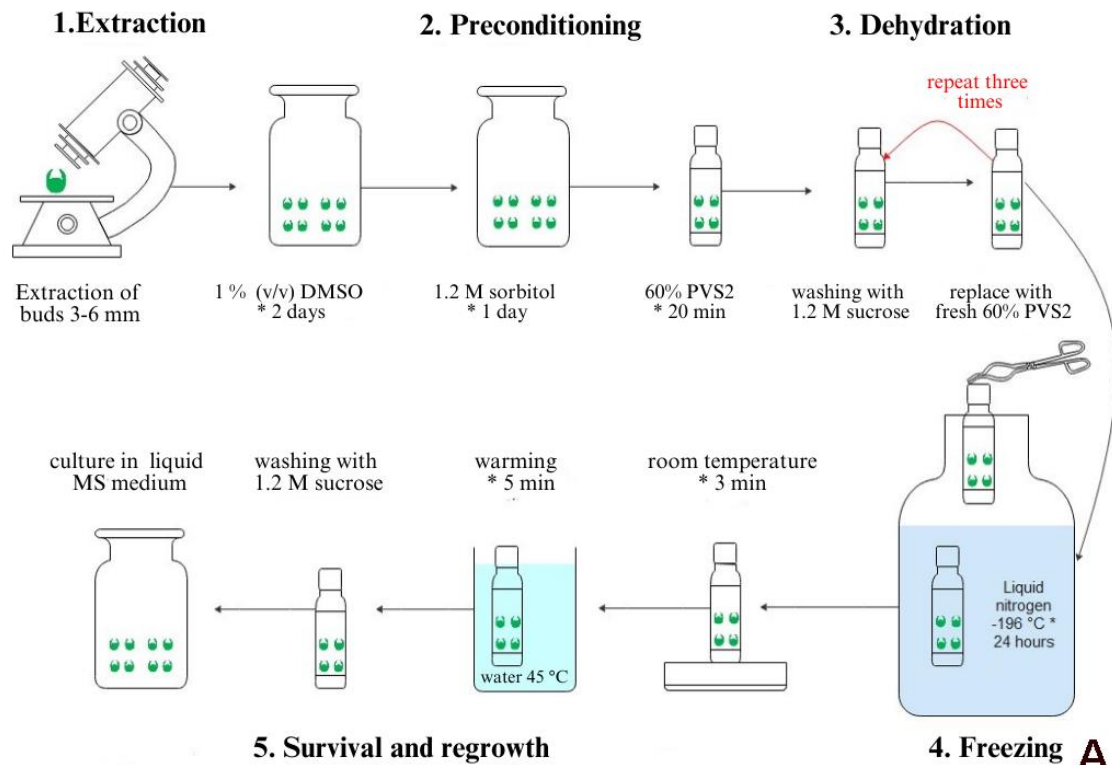
In vitro, 4-month-old seedlings were pre-cultivated in MS medium containing 2% (0.05 M) sucrose, solidified with 0.7% agar at a pH of 5.8 ± 0.02 , and autoclaved at 121°C for 20 minutes (110,000 Pa). These seedlings were grown under a 12-hour light and 12-hour dark photoperiod with 40 W fluorescent lamps, at an average temperature of $23 \pm 2^\circ\text{C}$ and a relative humidity of $65 \pm 7\%$.

From these seedlings, nodal segments of approximately 3-6 mm (Fig. 6D) with apical and axillary buds were aseptically extracted under a binocular microscope. These segments were used for two cryopreservation assays: i) vitrification (Fig. 1A); and ii) encapsulation-dehydration (Fig. 1B), in addition to a control (no cryopreservation treatment but freezing in liquid nitrogen). Solutions for both cases were prepared in liquid and semi-solid MS medium, with the pH adjusted to 5.8 ± 0.02 . Nodal segments extracted at all stages prior to freezing were cultivated under a 12-hour light and 12-hour dark photoperiod with 40 W fluorescent lamps, maintaining an average temperature of $23 \pm 2^\circ\text{C}$ and a relative humidity of $65 \pm 7\%$.

For the vitrification test (Fig. 1A; 6G), as a preconditioning step, the extracted segments were placed on filter paper submerged in a liquid medium containing the cryoprotectant at 1% (v/v) dimethyl sulfoxide (DMSO) and were kept at room temperature ($23 \pm 2^\circ\text{C}$) for 2 days. After this period, the segments were transferred to a semi-solid medium with 1.2 M sorbitol, and maintained at room temperature for 1 day. For dehydration, the segments were vitrified in a 60% solution of plant vitrification solution 2 (PVS2: 30% glycerol, 15% ethylene glycol, 15% DMSO, 0.4 M sucrose in $\frac{1}{2}$ MS) (Sakai et al. 1990) for 20 minutes. Subsequently, the segments were

washed with a discharge solution (culture medium plus 1.2 M sucrose), then placed again in a 60% solution of chilled PVS2, and washed three times with the discharge solution. After dehydration, 4 replicates (2 ml cryovials) containing 8-10 segments each were rapidly immersed in liquid nitrogen (-196°C) for 24 hours. A total of 35 nodal segments were evaluated.

In the encapsulation-dehydration process (Fig. 1B), extracted nodal segments were preconditioned by immersing them in a medium MS solution with 3% (v/v) DMSO and 0.75 M sucrose for two days. Subsequently, they were immersed in a medium MS solution without calcium containing 3% (w/v) sodium alginate, remaining submerged until completely enveloped in the alginate solution, a process completed within a maximum of 5 minutes. Following this, the segments were individually transferred to a medium MS solution with 100 nM CaCl_2 , facilitating the formation and solidification of alginate beads. These beads were then extracted for polymerization. For dehydration, they were dried in a laminar flow hood for 60 minutes at 25°C with 40% humidity. After dehydration, 4 sets of 8-10 dry beads (35 total) were placed in 2 ml cryovials and immediately submerged in liquid nitrogen for 24 hours.



A

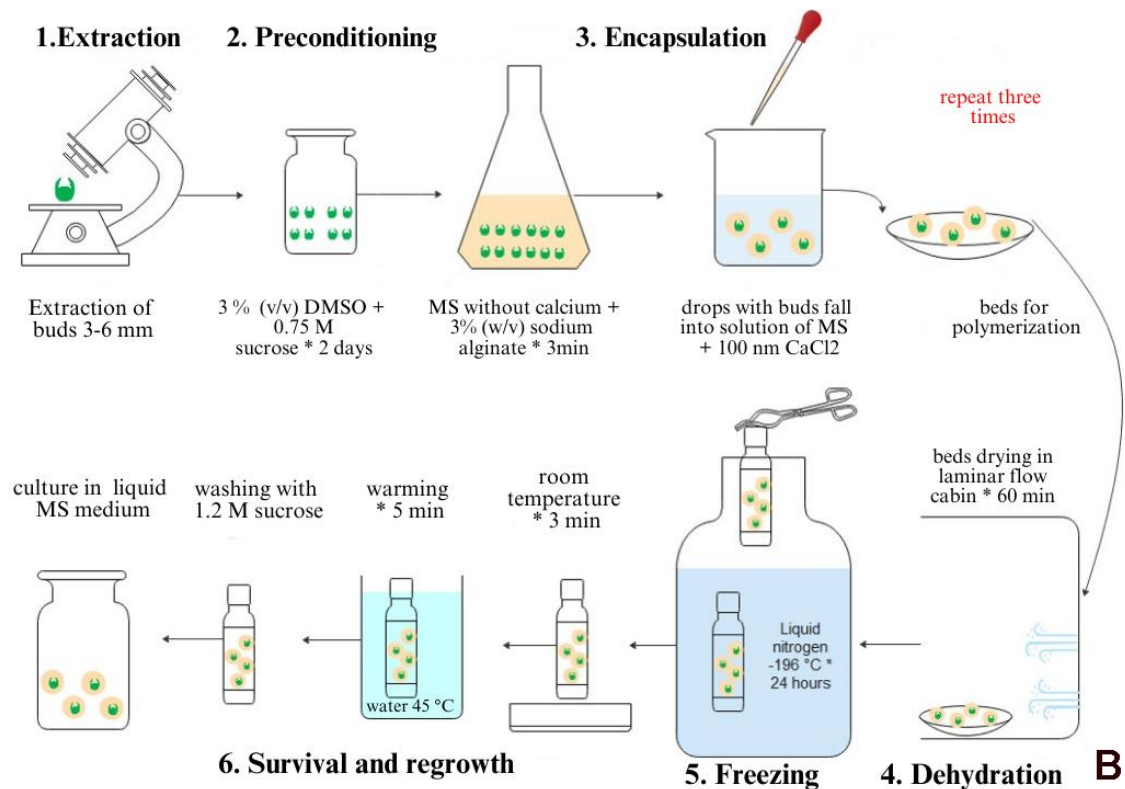


Figure 1 Flowchart of the cryopreservation process: A. Vitrification and B. Encapsulation-dehydration.

The control protocol was consistently applied in both experiments. Four-month-old seedlings were cultivated in MS medium with 2% sucrose. Nodal segments containing apical and axillary buds were aseptically extracted without prior treatment with cryoprotectants. Additionally, 4 repetitions (2 ml cryovials) each containing 8-10 segments were rapidly submerged in liquid nitrogen at -196 °C for 24 hours.

Recovery of nodal segments from both trials and the control involved removing the cryovials from liquid nitrogen. These cryovials, along with the segments, were kept at room temperature for 3 minutes before being immersed in a water bath at 45°C for 5 minutes

Data analysis

All results from the experimental treatments of slow-growth storage were analyzed using Generalized Linear Mixed Models (GLMMs) (Zuur et al. 2009). The binomial family with a logit link was employed to assess the impact of the type and concentration of the growth medium as well as the type and concentration of osmotic substances, and the storage time on mortality and root formation. These models proved suitable for handling binary data. To evaluate growth and the formation of new shoots, GLMMs with Gaussian and Poisson families were utilized, respectively. Significant differences between treatments were determined at the 12-month storage mark. These differences were analyzed using the least squares method and compared using a Tukey-Kramer test with a significance level of $\alpha = 0.05$. All calculations were performed in R version 3.6.3 (R Core Team, 2020) using the 'lme4' (Bates et al., 2015), 'lmerTest' using Satterthwait's method for approximation degrees of freedom (Kuznetsova et al. 2017), and 'lsmeans' (Russell 2016) packages. Regarding cryopreservation results, means $\pm$ standard deviation, and Multiple Regression Analysis are presented.

Results and Discussion

Responses to slow-growth storage

The modification of osmotic substances (mannitol, sorbitol, and sucrose) and the type and concentration of the culture medium were investigated to establish a protocol for the conservation of *C. officinalis* germplasm through slow growth. The effectiveness of in vitro conservation treatments was assessed, considering factors such as growth, shoot and root formation, and explant mortality, as documented in other species (Leva et al., 2013; Nogueira et al., 2014). The optimal method will be one that reduces growth (length, shoots, and roots) without compromising explant survival and with a lower risk of inducing genetic variations (Muñoz et al., 2019; Viegas et al., 2021). For this species, a storage period of 12 months without subcultures was evaluated. In other species, in vitro assessment of slow growth showed that explants had storage cycles ranging from 45 days (Hunter, 1986) to 28 months without subcultures (Gianní and Sottile, 2015; Oliveira and Ibrahim, 2022; Panis et al., 2020).

Growth

Explant growth was observed with all osmolytes and culture media treatments. However, growth was lower in all concentrations of osmotic substances when compared to the different concentrations and types of culture media during the 12 months of culture (Fig. 2).

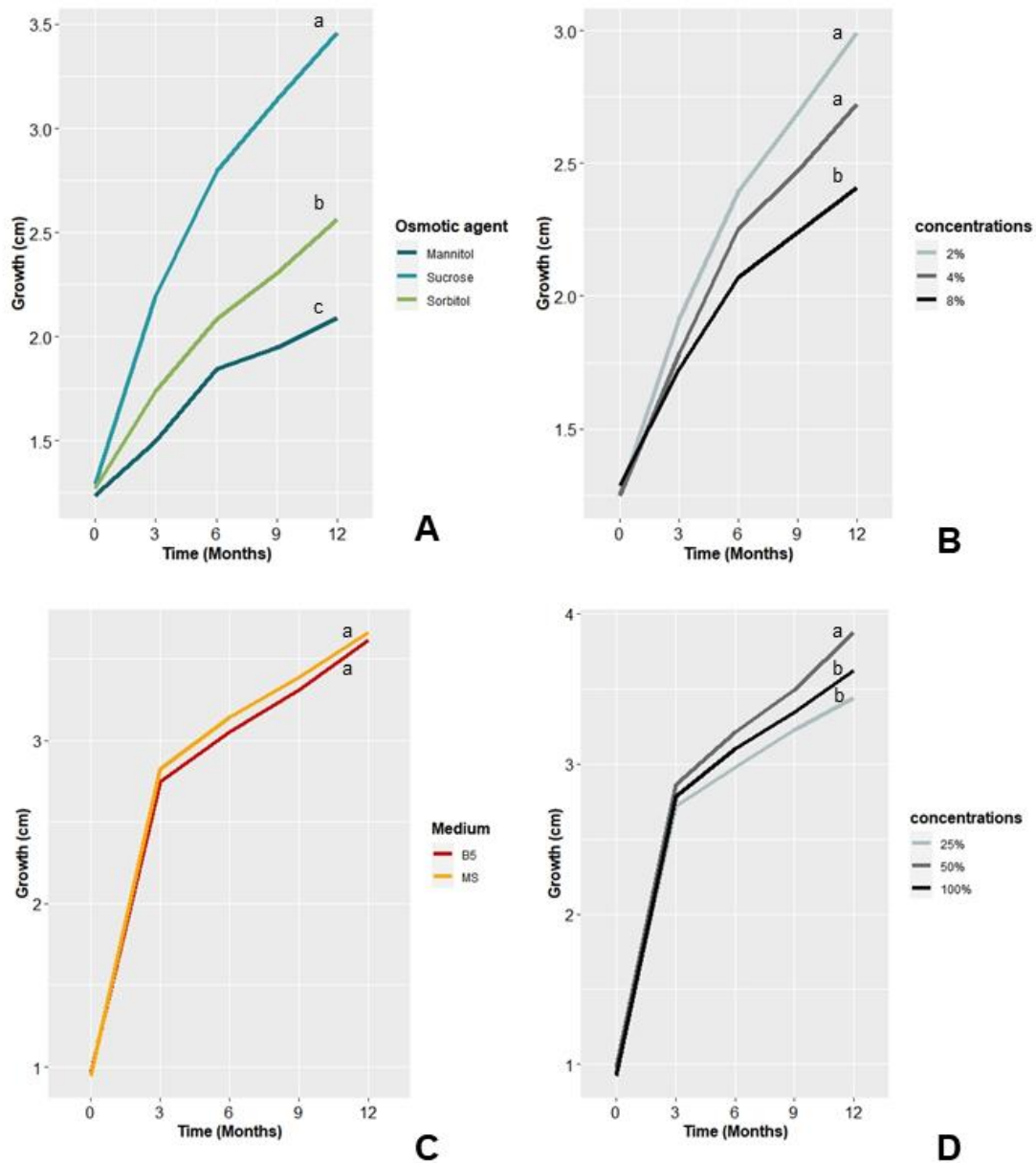


Figure 2 Groups of concentrations of osmotic substances, culture media at different concentrations, and storage time (0, 3, 6, 9, 12 months) on the growth of explants: A. Types of osmotic substances; B. Concentrations of osmotic substances (2%, 4%, and 8%); C. Types of culture media; D. Concentrations of culture media (25%, 50%, and 100%). At twelve months of storage, values followed by the same letter in the same figure are not significantly different at the 0.05 probability level after multiple contrasts.

In the osmotic substance assay, both the different substances and their concentrations significantly affected the explant development, as evidenced in Table 2. When considering all evaluated concentrations, the three osmotic substances were categorized into three groups with statistically significant differences, as depicted in Figure 2A. Notably, mannitol proved to be the most effective osmotic substance in inhibiting growth, as observed in Figure 2A and 6C. Additionally, the concentration of osmotic substances was segregated into two groups with statistically significant differences. As the concentration of these substances increased, a proportional decrease in explant growth was recorded, as illustrated in Figure 2B.

Table 2 Summary table of the GLMMs, using Satterthwait's method, for growth, shoot formation, root formation and mortality in response to different osmotic substances and osmotic substance concentrations in different time periods.

Factor	Variation source	Estimate	Standard Error	<i>p</i>	
Growth					
Osmotic substances	Time	0.11696	0.00248	< 2.00E-16	***
	Mannitol	1.14E+05	0.03349	< 2.00E-16	***
	Sucrose	0.84322	0.02562	< 2.00E-16	***
	Sorbitol	0.26572	0.02584	< 2.00E-16	***
Concentration	2%	1.14E+05	0.03349	< 2.00E-16	***
	4%	-0.07262	0.02655	0.00629	**
	8%	-0.25956	0.02613	< 2.00E-16	***
Shoot formation					
Osmotic substances	Time	0.26232	0.00896	< 2e-16	***
	Mannitol	1.60E+05	0.12049	< 2e-16	***
	Sucrose	2.18E+05	0.09258	< 2e-16	***
	Sorbitol	-0.23809	0.09337	0.0108	*
Concentration	2%	1.60E+05	0.12049	< 2e-16	***
	4%	0.41927	0.09593	1.31e-05	***
	8%	0.55853	0.09442	3.92e-09	***
Root formation					
Osmotic substances	Time	0.3421	0.0232	< 2e-16	***
	Mannitol	-9.71E+04	0.6178	< 2e-16	***
	Sucrose	7.28E+04	0.5392	< 2e-16	***
	Sorbitol	27,676	0.5290	1.68e-07	***
Concentration	2%	-9.71E+04	0.6178	< 2e-16	***
	4%	1.23E+04	0.1986	5.25e-10	***
	8%	1.52E+04	0.2063	1.62e-13	***
Mortality					
Osmotic substances	Time	0.65549	0.03239	< 2e-16	***
	Mannitol	-6.51E+05	0.35950	< 2e-16	***
	Sucrose	-1.37E+05	0.18231	5.86e-14	***
	Sorbitol	-105,304	0.18170	6.81e-09	***
Concentration	2%	-6.51E+05	0.35950	< 2e-16	***
	4%	-0.12668	0.19038	0.506	
	8%	1.83E+05	0.19538	< 2e-16	***

In the culture media assay, both the MS medium and the 100% medium concentration did not significantly impact growth. Conversely, the remaining factors led to a substantial increase in growth, as outlined in Table 2. The multiple contrast test did not reveal significant disparities in growth between the B5 and MS media, as depicted in Figure 2C. Regarding the medium concentration, a significant difference was observed in two groups. Specifically, the 50% concentration exhibited slightly better growth, as illustrated in Figure 2D.

Shoot formation

The formation of new shoots was influenced by the type of osmotic substance, the different concentrations and the evaluation time (Table 2). The osmotic substances formed two significantly different groups (Fig. 3A). Shoot formation was higher with sucrose, whereas it was low when the explants were cultured in mannitol (Fig. 6C) or sorbitol (Fig. 6E). Among the different concentrations tested, two significantly different groups were observed. The 2% concentration showed low shoot formation. Osmotic substances such as sorbitol reduce mineral uptake by changing the osmotic pressure, which inhibits growth and new shoot formation (Ambrožič-Dolinšek et al. 2022; Heba et al. 2022).

The generation of new shoots was influenced by the type of osmotic substance, various concentrations, and storage duration (Table 2). Osmotic substances formed significantly distinct groups, as depicted in Figure 3A. Shoot proliferation was notably higher in the presence of sucrose, while it was limited in explants cultivated in mannitol or sorbitol. Different concentrations of osmotic substances revealed two significantly distinct groups, with the 2% concentration associated with poor shoot generation. Osmotic substances, such as sorbitol, impact mineral absorption by modifying osmotic pressure, resulting in the inhibition of growth and the formation of new shoots (Ambrožič-Dolinšek et al., 2022; Heba et al., 2022).

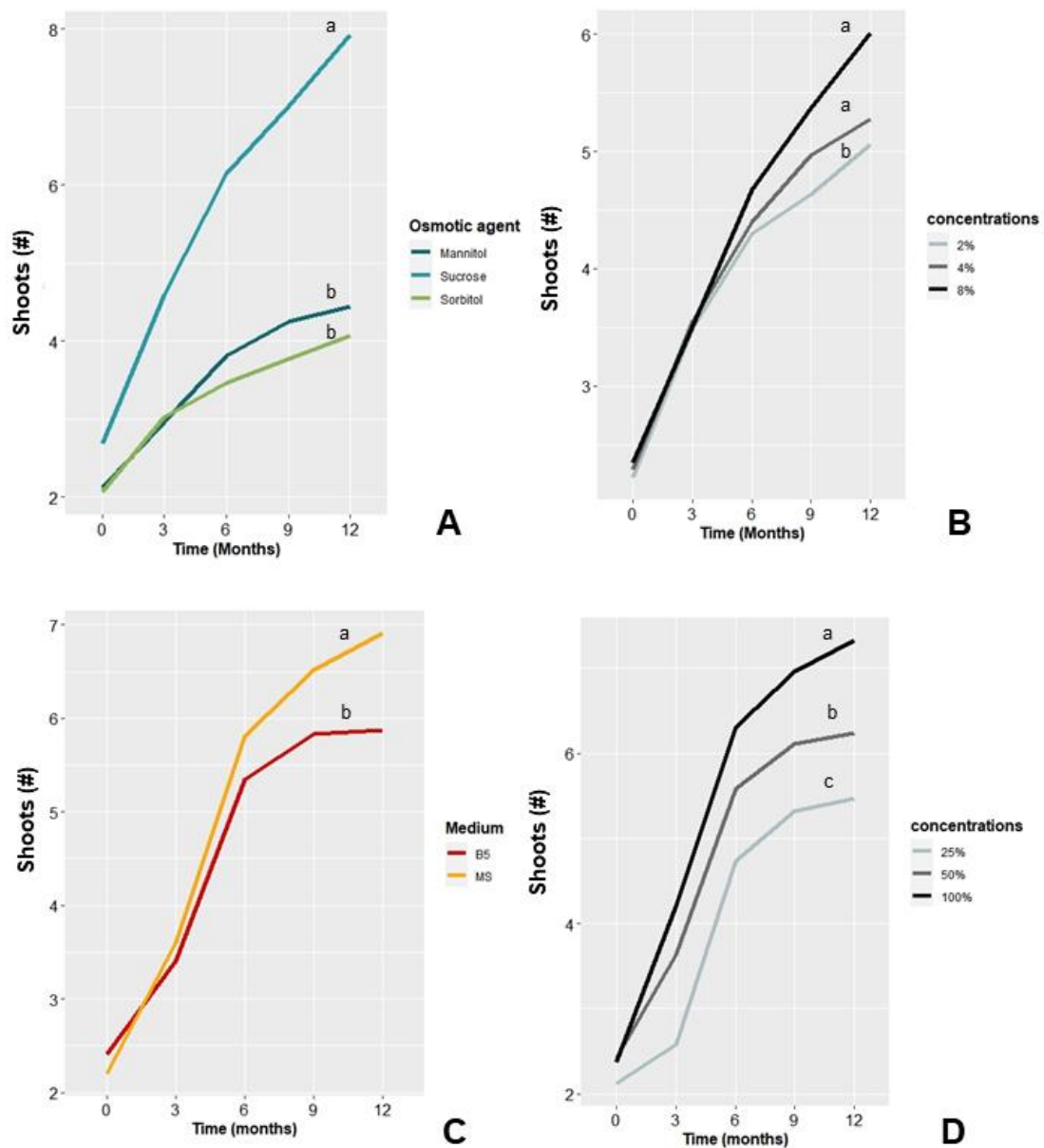


Figure 3 Groups of concentrations of osmotic substances, culture media at different concentrations, and storage time (0, 3, 6, 9, 12 months) on the number shoot formed: A. Types of osmotic substances; B. Concentrations of osmotic substances (2%, 4%, and 8%); C. Types of culture media; D. Concentrations of culture media (25%, 50%, and 100%). At twelve months of storage, values followed by the same letter in the same figure are not significantly different at the 0.05 probability level after multiple contrasts.

In relation to the culture medium, various factors, such as the type of medium and concentrations, played a crucial role in shoot generation, as detailed in Table 2. Statistically significant differences were observed among the different media used. After a period of 12 months, a notable decrease in the number of shoots was recorded in the B5 medium, as illustrated in Figure 3C. The three concentrations of both culture media exhibited significant variations in the ability to induce shoots (Fig. 6F). It is important to note that the 25% concentration was associated with the lowest rate of shoot formation, as observed in Figure 3D.

Root formation

To illustrate the influence of different osmotic substances, culture media, and concentrations on root formation and mortality, we present the percentage of responses for each individual, calculated from binary data (1: presence/0: absence of roots; 1: alive/0: dead).

All factors demonstrated an impact on root formation. Root formation was observed predominantly in the later months (9 and 12 months). A significant difference was identified among the three osmotic substances. The highest percentage of rooting was recorded across all evaluated sucrose concentrations. Mannitol presence exhibited the lowest percentage of rooting (Fig. 4A). The 2% concentration, the lowest among the three osmotic substances, significantly influenced root formation, reducing it (Table 3).

Root formation was observed at a higher percentage in the presence of sucrose and lower for sorbitol and mannitol. Matos et al. (2020) reported similar results in the *Amburana cearensis* cultivar. Water stress caused by the presence of mannitol and sorbitol does not stimulate root development (Gopal and Chauhan 2010), as it reduces the turgor pressure necessary for cell division, as observed in *Psychotria ipecac* (Urrea-Trujillo et al. 2017).

Table 3 Summary table of the GLMMs, using Satterthwait's method, for growth, shoot formation, root formation and mortality in response to different medium and medium concentration in different time periods.

Variation source		Estimate	Standard error	<i>p</i>	
Growth					
Medium	Time	1.98E+02	5.36E+00	< 2e-16	***
	MS	6.43E+01	4.60E+01	0.163	
	B5	1.47E+03	7.93E+01	< 2e-16	***
Concentration	25%	1.47E+03	7.93E+01	< 2e-16	***
	50%	2.47E+02	5.84E+01	2.6e-05	***
	100%	6.19E+01	5.49E+01	0.259	
Shoot formation					
Medium	Time	3.62E+02	1.21E+01	< 2e-16	***
	MS	5.10E+02	1.04E+02	9.85e-07	***
	B5	1.61E+03	1.68E+02	< 2e-16	***
Concentration	25%	1.61E+03	1.68E+02	< 2e-16	***
	50%	8.11E+02	1.32E+02	9.42e-10	***
	100%	1.40E+03	1.24E+02	< 2e-16	***

Root formation					
Medium	Time	0.294481	0.001181	<2e-16	***
	MS	-1,130,255	0.001183	<2e-16	***
	B5	-3,534,686	0.001183	<2e-16	***
Concentration	25%	-3,534,686	0.001183	<2e-16	***
	50%	-0.626193	0.001183	<2e-16	***
	100%	-0.904518	0.001183	<2e-16	***
Mortality					
Medium	Time	0.59099	0.03754	< 2e-16	***
	MS	0.02983	0.16900	0.85987	
	B5	-595,670	0.39784	< 2e-16	***
Concentration	25%	-595,670	0.39784	< 2e-16	***
	50%	-0.60340	0.20097	0.00268	**
	100%	-140,488	0.21771	1.1e-10	***

In relation to the type and concentration of the culture medium, we observed that all factors influenced root formation (Table 3). Significant differences were evident between the B5 and MS media. It's noteworthy that the MS medium exhibits a lower percentage of root formation (Fig. 4C). Additionally, the three concentrations used show significant differences among themselves. The lowest rooting percentage was recorded in both media at 100% (Fig. 4D). Overall, for most concentrations, the rooting percentage tends to increase over time and is higher as the percentage of available nutrients decreases (Figure 4D).

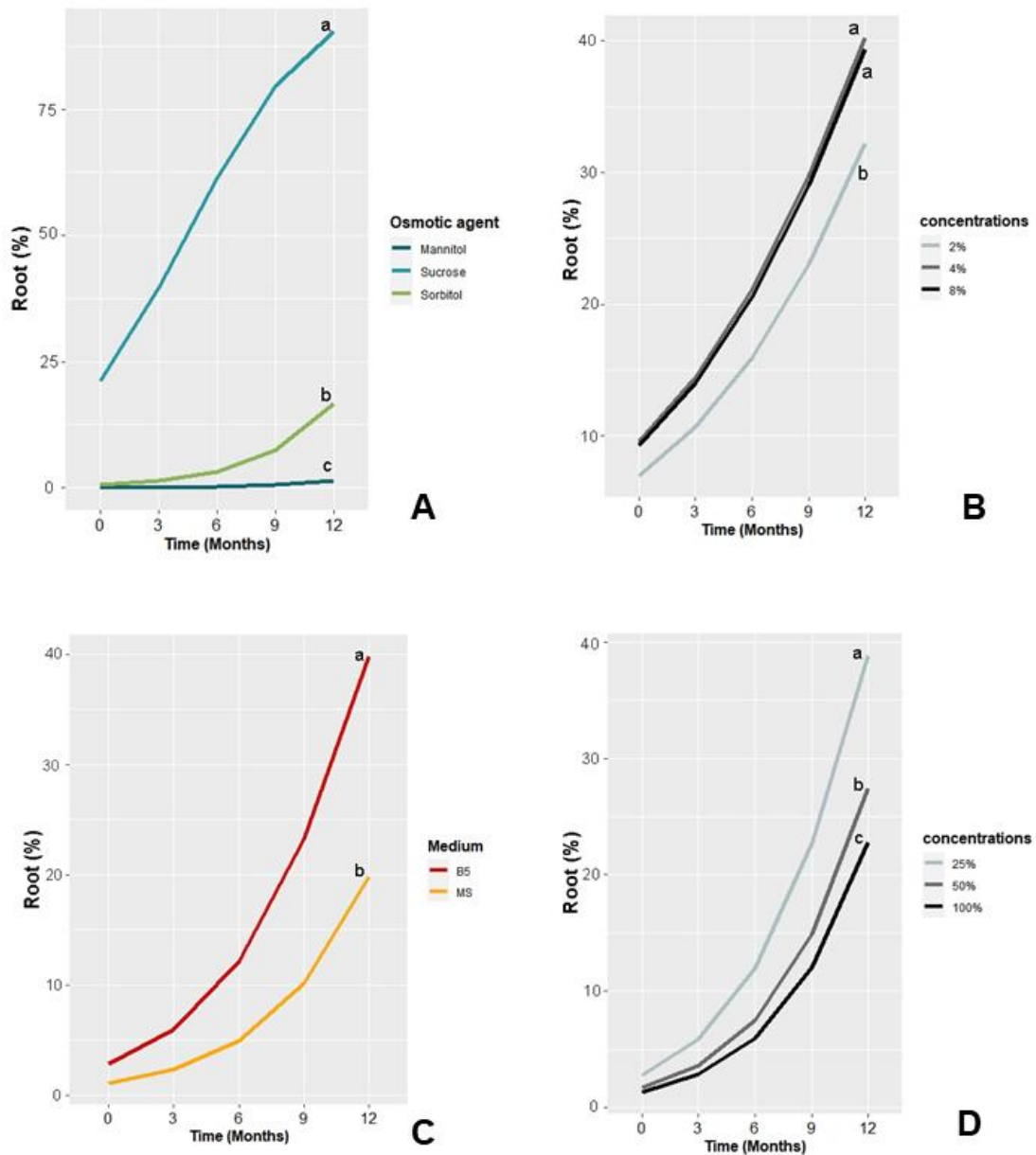


Figure 4 Groups of concentrations of osmotic substances, culture media at different concentrations, and storage time (0, 3, 6, 9, 12 months) on root formation percentage: A. Types of osmotic substances; B. Concentrations of osmotic substances (2%, 4%, and 8%); C. Types of culture media; D. Concentrations of culture media (25%, 50%, and 100%). At twelve months of storage, values followed by the same letter in the same figure are not significantly different at the 0.05 probability level after multiple contrasts.

The root proliferation was higher when nutrient concentrations in the growth medium were reduced, especially evident in the B5 medium at 25% concentration. Overall, explants exposed to different nutrient concentrations increased their roots over storage time. Root formation serves as a survival mechanism against water and nutrient deficits; plants develop root systems to enhance their ability to seek and absorb these elements, enabling adaptation to stressful conditions (Castilla-Valdés et al. 2020). This phenomenon has been observed in *Triticum aestivum* plants cultivated in the last months of the growing season when water deficit is more significant (Volgger et al. 2010).

Mortality

The evaluated factors, such as the type and concentration of osmotic substances, significantly impact mortality (Table 2). Specifically, mannitol shows a notable difference compared to sorbitol and sucrose, exhibiting the highest mortality percentage (Fig. 5A). Regarding concentration, 8% differs significantly from 4% and 2%, presenting the highest mortality percentage (Fig. 5B).

Concerning the culture media, only B5 influences mortality according to the GLMM analysis. However, both culture media are grouped into a single category (Fig. 5C). As for the impact of different concentrations of the culture medium on explant mortality, this is significant at three levels: 25%, 50%, and 100% (Table 3). The highest mortality percentage is observed at concentrations of 25% and 50% (Fig. 5D; 6B).

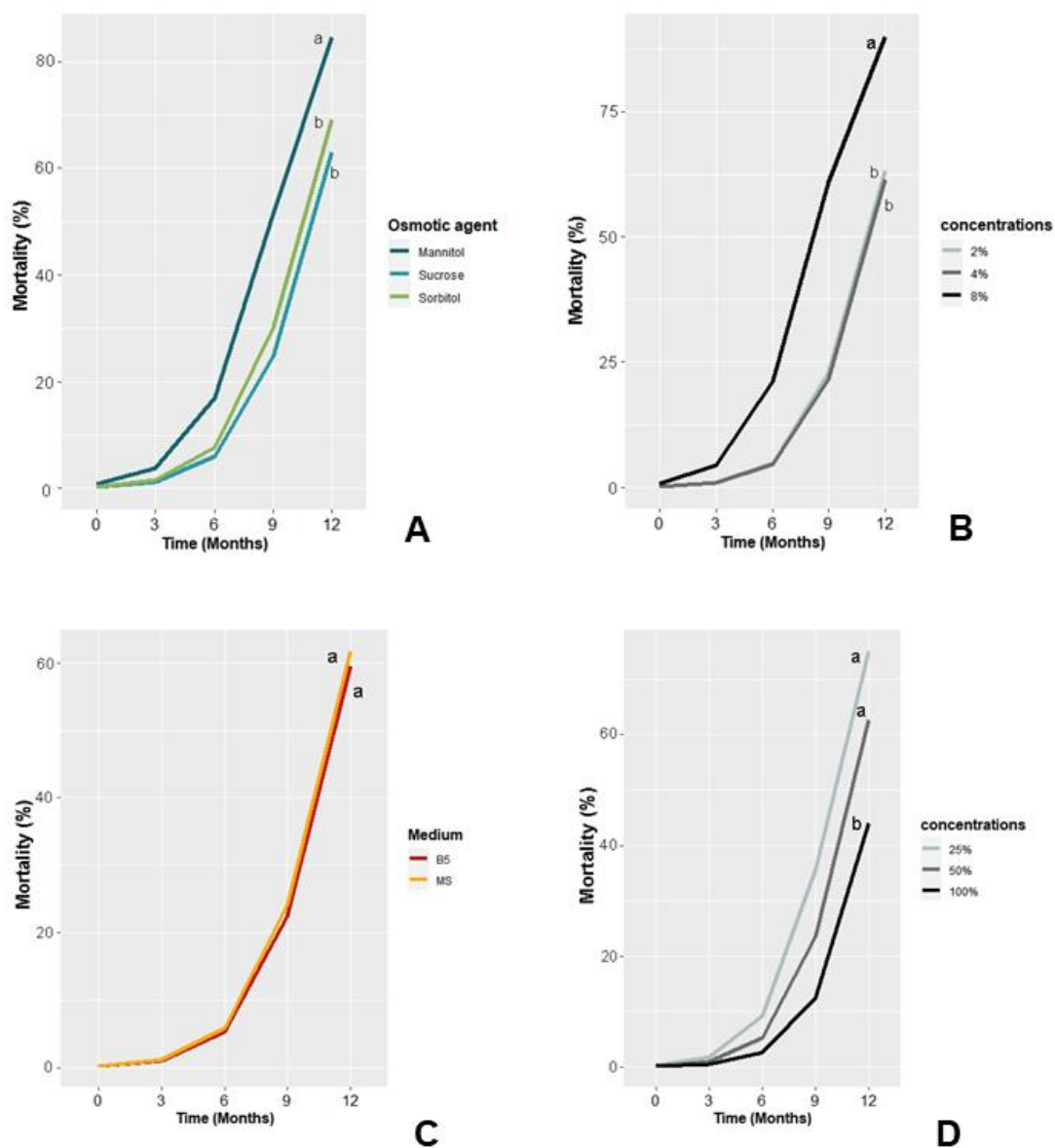


Figure 5. Groups of concentrations of osmotic substances, culture media at different concentrations, and storage time (0, 3, 6, 9, 12 months) in the percentage of mortality of explant: A. Types of osmotic substances; B. Concentrations of osmotic substances (2%, 4%, and 8%); C. Types of culture media; D. Concentrations of culture media (25%, 50%, and 100%). At

twelve months of storage, values followed by the same letter in the same figure are not significantly different at the 0.05 probability level after multiple contrasts.

In general, the inclusion of osmotic substances such as sucrose, mannitol (Silva and Scherwinski-Pereira, 2011; Akdemir et al., 2013), and sorbitol (Díaz-Narváez et al., 2015; Elhomosany and El-Wahab, 2020; Petrova et al., 2020; Benelli et al., 2022; Khan et al., 2022), as well as the reduction of nutrient concentration (Mohanty et al., 2013; Castilla-Valdés et al., 2020) in the culture medium, influence the subculturing interval and storage time.

In *C. officinalis*, all explants exhibited chlorosis after six months of storage, followed by necrotic lesions in leaves and stems, culminating in the high mortality of the explants in several cases. Mortality gradually increases in all explants subjected to treatments with osmotic substances and culture media during the twelve-month storage period. Specifically, in the presence of mannitol, the highest levels of mortality are observed, supporting similar observations in *Uncaria tomentosa* (Cruz-Mora et al., 2011) and *Arnica montana* (Petrova et al., 2020), where tissue oxidation and death are manifested. A determining factor in mortality is the concentration of the osmotic substance, especially in the case of mannitol; high concentrations are associated with increases in mortality, consistent with similar findings reported by Bello-Bello et al. (2014) and Khan et al. (2022). At the end of the storage period, sucrose proves to be the osmotic substance with the lowest percentage of mortality, followed by sorbitol.

In the case of explants grown in nutrient-deficient media, minimal changes were observed that contributed to slow growth, accompanied by deficiency symptoms and progressive mortality over twelve months. The highest mortality rate was recorded at the lowest concentration (25%). Similar findings have been documented in crops like *Gypsophilla petraea* and *Dianthus callizanus*, cultivated in media at 1/4 and 1/10, respectively, for twelve months (Catana et al., 2010). The modification of the medium concentration plays a crucial role in reducing cell division, thereby limiting proliferation and shoot growth (Benelli et al., 2022; Faisal and Alatar, 2019). Consequently, adjusting medium concentrations is possible without compromising explant survival during storage.

In the context of *C. officinalis*, osmotic substances act as growth retardants by inducing osmotic stress in explants. However, each substance exhibits distinct responses affecting conservation without subcultures. Mannitol showed the highest mortality rate, coupled with reduced shoot growth, budding, and a low rooting percentage. In contrast, in the presence of sucrose, mortality rates were lower, while growth, shoot formation, and rooting percentage were higher. Sorbitol, as an osmotic regulator, proved effective in reducing mortality and slowing the growth and development of new shoots during the twelve-month storage period. It's noteworthy that sorbitol induces less severe osmotic stress than other osmotic substances without contributing to plant toxicity or inducing anomalies, thereby promoting higher survival rates (Hassan et al., 2014; Nasiruddin and Rafiul, 2018).

It is crucial to note that explants exposed to both sorbitol and mannitol exhibited, in many cases, weak shoots with reddish hues, accompanied by signs of chlorosis primarily affecting the leaves. This adverse response could be attributed to potential nutrient deficiencies in the medium and induced stress, a phenomenon observed in various pear genotypes (Reed et al., 2013). Consequently, after the conservation period, it becomes imperative to subject the explants to a recovery phase. This additional step is essential for enhancing the growth

rate of the explants, as supported by previous studies in plant genetics (Lata et al., 2010; Cordeiro et al., 2014; Ambrožič-Dolinšek et al., 2022). The results indicate that sorbitol can be effective as an osmotic substance for the short-term conservation of *C. officinalis* by inducing slow growth of the explants. This strategic approach can be valuable in situations where a controlled deceleration of development is required to achieve successful preservation.

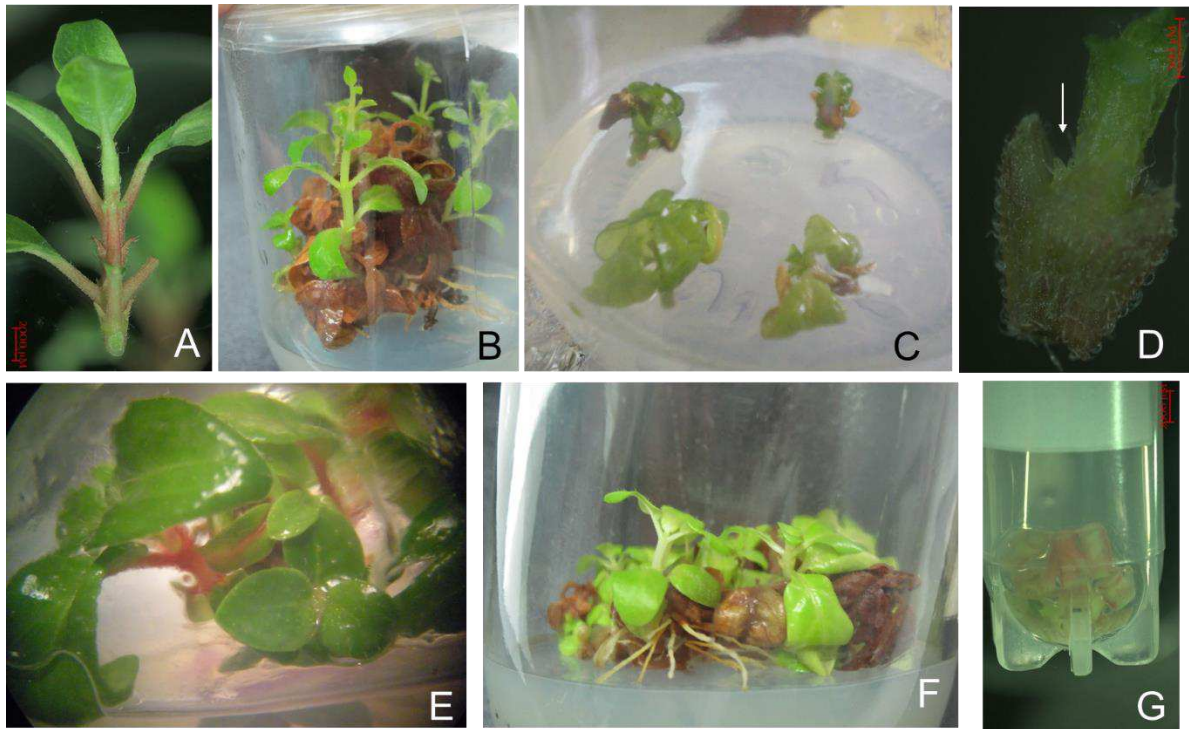


Figure 6. In vitro conservation: A. Four-month-old in vitro seedling, B. Shoots cultured in MS medium with several dead explants and root development. C. Explants cultured in medium with mannitol, showing limited shoot formation and growth, without the presence of roots. D. Axillary and apical buds before cryopreservation. E. Explants cultured in medium with sorbitol, with limited shoot formation and root development. F. Explants in B5 medium, with abundant sprouting, root presence, and some dead explants. G. Buds subjected to vitrification before freezing in liquid nitrogen.

Cryopreservation

Segments with axillary and apical buds were used to establish a cryopreservation protocol, employing the methods of vitrification and encapsulation-dehydration as cryoprotection processes. These procedures are crucial to prevent potential damages associated with freezing, leading to an improvement in the post-cryopreservation phase, as indicated by Bajaj (1998). In the tissues of *C. officinalis*, the effectiveness of these procedures was evaluated during the recovery period, analyzing the growth and survival of shoots over 45 days following freezing in liquid nitrogen.

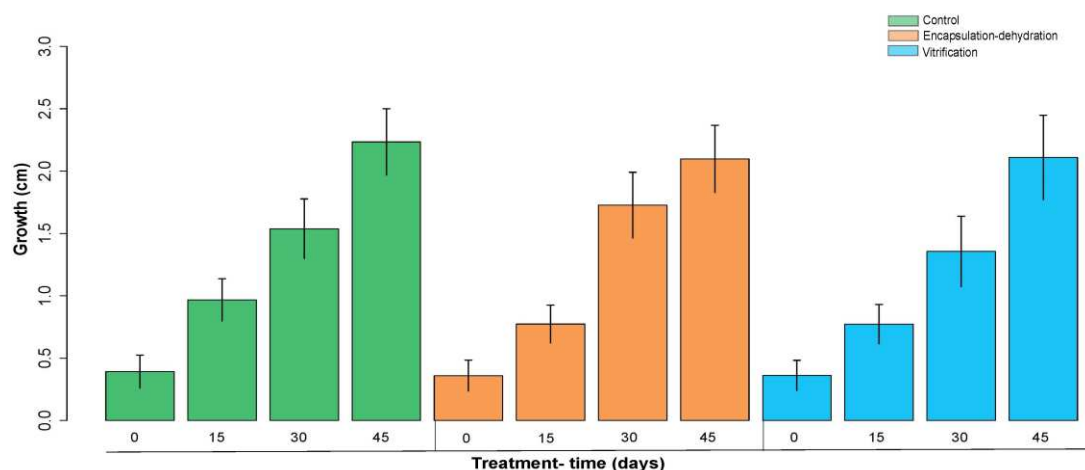


Figure 7 Growth of the explants (mean and standard deviation) throughout the 45-day recovery process.

Statistically significant differences were observed among the various treatments, as evidenced in Table 4, concerning the growth factor. In the encapsulation-dehydration procedure, the explants displayed the lowest growth rate after cryopreservation, as illustrated in Figure 7. Conversely, a significant increase was recorded in the control treatments, followed by the vitrification process.

Table 4. Summary of the Multiple Regression Analysis explaining explant growth and mortality after Encapsulation-Dehydration and Vitrification.

Factor	Estimate	Standard error	Pr (< t)
Growth			
Encapsulation-Dehydration	0.05	0.02	0.0478 *
Vitrification	-0.12	0.03	6.55e-06 ***
Time	0.04	0.00	9.63e-15 ***
Control	0.38	0.02	<2e-16 ***
Mortality			
Encapsulation-Dehydration	9.5	3.34	0.004690 **
Vitrification	32.5	3.34	<2e-16 ***
Time	0.63	0.08	4.1e-14 ***
Control	-11.2	2.99	0.000201 ***

At 45 days of evaluation, it was observed that explants untreated with methods of vitrification or encapsulation-dehydration (control group) exhibited the lowest mortality rate at 4%. In comparison, those subjected to encapsulation-dehydration showed a mortality rate of 24%, while those treated using the vitrification method displayed the highest rate at 58% (Fig. 8). Control explants were exclusively cultured in a medium enriched with 2% sucrose before freezing. Sucrose has proven to be an effective cryoprotectant in various previous studies (Bajaj 1988; Bekheet 2011; Panis et al. 2020; Ruta et al. 2020).

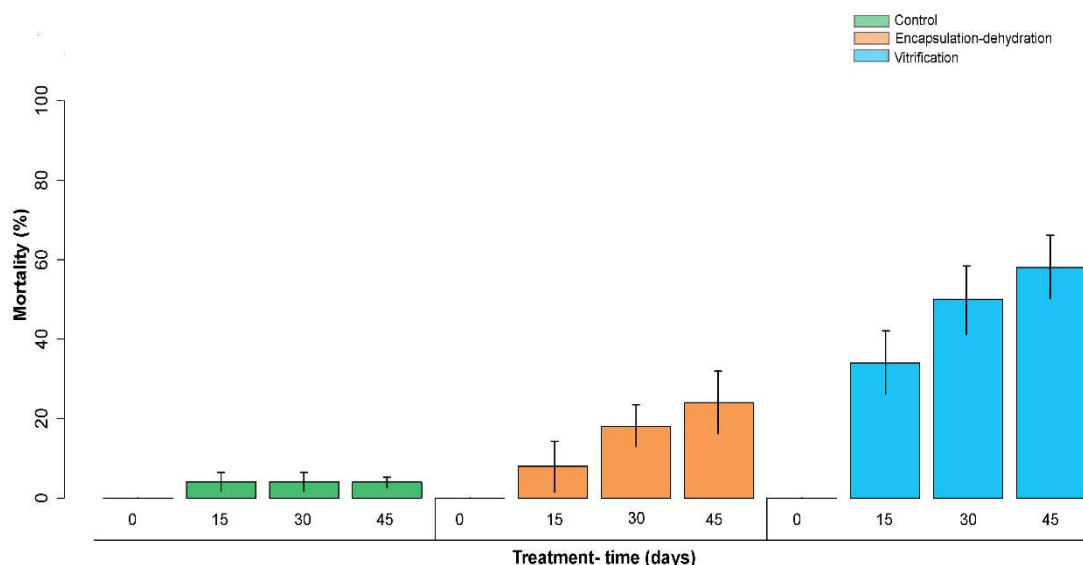


Figure 7 Mortality of the explants (mean and standard deviation) throughout the 45-day recovery process.

Long-term tissue preservation, through storage in liquid nitrogen for several years, is another alternative for germplasm conservation. In this study, we utilized bud segments treated with vitrification and encapsulation-dehydration techniques. All nodal segments for the cryoconservation treatments, including the control group, were extracted from seedlings that were pre-cultured in a medium with sucrose. It was observed that those explants (control) not subjected to vitrification and encapsulation-dehydration processes had higher survival and recovery percentages after freezing. To achieve tissue dehydration and ensure a high survival percentage, it is crucial for the culture medium to have a high concentration of sucrose (Gonzalez-Arno et al., 2008). Similar observations have been made in *Morus alba*, where adding sucrose to the culture medium proved effective in promoting explant survival and regrowth after freezing (Arias et al., 2012).

Low sucrose concentrations do not impact tissue viability and may assist in inducing tolerance to more severe dehydration conditions (Osorio et al., 2011). The beneficial effect of sucrose is attributed to its accumulation in cells and partial metabolism (Barraco et al., 2014). Maintaining a water content between 10-20% is crucial for subsequent recovery (Gonzalez-Arno et al., 2008). Sucrose acts as an osmoprotector; its concentration enhances tolerance to severe dehydration conditions in tissues exposed to cold (Zamecnik et al., 2021; Kim and Popova, 2023; Zhang et al., 2023).

Although a lower percentage of tissues subjected to the encapsulation-drying process managed to survive freezing, the compounds DMSO and sucrose used in this technique exerted a significant influence on this outcome. Similar findings have been observed in *Rabdosia rubescens*, where encapsulation-drying protects intracellular components from damage caused by water crystallization during freezing, demonstrating effectiveness with an 85% recovery of cryopreserved meristems (Ai et al., 2012), 5-18% recovery in pistachios (Akdemir et al., 2013), and a 57% recovery with 100% regeneration in apples (Li et al., 2014). Crucial factors in cryopreservation include the type and size of the explant, with a significant impact on recovery. In this study, nodal segments with apical and axillary buds were used, characterized by containing the meristem protected by leaf primordia. This protective effect has also been observed in apples (Li et al., 2014) and other species (O'Brien et al., 2020; Zhang et al., 2023; Popova et al., 2023).

Finally, the vitrification technique proved ineffective as a cryoprotective system for *C. officinalis* explants, recording the lowest recovery percentage. Similarly, pre-cultivated *C. ledgeriana* callus for 3 days in 5% PVP medium, treated with cryoprotectants and submerged in liquid nitrogen, did not survive thawing (Hunter, 1986). Vitrification induces viscosity in intracellular contents and inhibits molecular rearrangement of water (Gonzalez-Arnan et al., 2008). Direct exposure of tissues to cryoprotectants during vitrification is critical, as high concentrations and prolonged exposure can be toxic (Gonzalez-Arnan et al., 2008; Le Bras et al., 2014). The efficacy of this technique is linked to the exposure time to PVS2, aiming to reduce tissue stress and mortality (Sulong et al., 2018).

In summary, tissues subjected to control culture followed by encapsulation-drying treatment favored higher survival percentages of explants after freezing.

Conclusions

This study represents the first investigation into the in vitro preservation of *C. officinalis* tissues. To preserve tissues for short periods, the cultivation of explants in a medium with sorbitol as an osmotic agent demonstrates benefits, including reduced mortality rates and a deceleration in growth, as well as the development of new shoots and roots. Despite the production of weak shoots with signs of chlorosis, their recovery is evident when cultivated in a fresh medium. Regarding cryopreservation, further testing is required, but preliminary results are presented that are valuable for the long-term cryogenic preservation of tissues. It is crucial to consider that tissues should be previously cultured in a sucrose medium, or alternatively, an encapsulation-dehydration treatment can be applied to nodal segments.

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