



Disinfection methods to obtain axenic cultures of *Posidonia oceanica* seedlings germinated from beach cast fruits

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Abstract

The regeneration of seagrass meadows through restoration efforts relies heavily on transplantable plant material, which is currently sourced from natural populations. However, the availability of this material is limited due to the lack of established axenic micropropagation protocols, highlighting the need for further research in this area. A major challenge in *Posidonia oceanica* micropropagation is explant disinfection, which is essential for maintaining axenic cultures and enabling the use of media and treatments that promote tissue multiplication. This study evaluated the effectiveness of various disinfection treatments on the establishment of axenic cultures of *P. oceanica* seedlings germinated from beach-cast fruits at different developmental stages, providing a sustainable alternative to harvesting from natural meadows. Results showed that axenic culture success depended on seedling age, with 2-month-old seedlings achieving the highest survival ($80 \pm 13\%$ at 40 weeks), compared to newly released seeds ($27 \pm 12\%$ at 48 weeks) and 6-month-old seedlings ($15 \pm 8\%$ at 12 weeks). The most effective disinfection protocol involved soaking 2-month-old seedlings in 0.5% sodium hypochlorite for 5 min. These findings lay the foundation for biotechnological approaches to *P. oceanica* restoration that reduce dependence on natural donor populations.

Keywords Seagrass · Seedlings · Micropropagation · Transplant, regeneration, restoration.

Introduction

Unlike terrestrial plant species, aquatic angiosperms such as seagrasses have been poorly studied in the context of micropropagation, despite their vital role in coastal ecosystems. Seagrass meadows are biodiversity hotspots, supporting over 1,000 associated taxa, many of which are rare, meadow-exclusive, or of commercial interest (Hemminga and Duarte 2000; Larkum et al. 2006; Urra et al. 2013; Padiglia et al. 2018). In addition, they help mitigate global warming by storing large amounts of carbon, approximately $140 \text{ g C m}^{-2} \text{ yr}^{-1}$ in sediments (Fourqurean et al. 2012), and more than 770 kg C m^{-2} in ancient mats (Serrano et al. 2016). Oxygen production in seagrass meadows can exceed that of tropical forests, reaching up to $408 \text{ mmol O}_2 \text{ m}^{-2} \text{ day}^{-1}$ under optimal conditions (Berg et al. 2019). Seagrasses can also act as natural filters cleaning their waters from contaminants, stabilizing sediments, and protecting coasts from erosion by currents and waves (Costanza et al. 2014). Despite their ecological significance, seagrass meadows are globally

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threatened, especially due to local human-associated disturbances and climate change (Orth et al. 2006; Waycott et al. 2009).

The seagrass *Posidonia oceanica* (L.) Delile, a slow-growing clonal species (1–6 cm rhizome yr^{-1} ; Marbà and Duarte 1998), is particularly vulnerable, often requiring decades to recover from disturbance (Kendrick et al. 2005; Di Carlo et al. 2005). Efforts to restore this species are crucial for accelerating its recovery, but they rely on limited transplantable resources such as seeds, seedlings and rhizome fragments, usually obtained from natural populations. Moreover, seed availability is highly restricted due to the irregular and unpredictable nature of flowering and fruiting events (e.g., Balestri 2004; Diaz-Almela et al. 2006). Developing micropropagation methods for *P. oceanica* seeds and seedlings could address these limitations by enabling long-term storage and flexible use of plant material.

Successful plant micropropagation requires species-specific disinfection protocols, as well as the development of appropriate culture media and acclimation procedures. Advancing through each of these stages requires an effective protocol for the previous phase. Seagrasses host diverse microbial communities on their tissues, making them prone to contamination. Inadequate disinfection can lead to microbial overgrowth in nutrient-rich media, inhibiting explant development. As a result, most published evidence on seagrass micropropagation focuses on the few species for which disinfection protocols have been successful. Nevertheless, the available literature on disinfection methods for seagrass seeds is diverse, reflecting a range of approaches and varying levels of effectiveness.

For the genus *Posidonia*, only four studies have tested disinfection methods using fruits or seeds of *P. oceanica* (Loquès et al. 1990; Balestri et al. 1998) and *coriacea* (Henry 1998; Wilson and Bennett 2008). Loquès et al. (1990) failed to establish axenic cultures with *P. oceanica* seeds, whereas Balestri et al. (1998) achieved 75% survival after 10 months. For *P. coriacea*, Henry (1990) reported 32% survival under axenic conditions after 16 weeks, while Wilson and Bennett (2008) obtained 72% survival after disinfection and basal medium culture, maintaining seedlings in vitro for up to two years. The most successful attempts to obtain axenic cultures from seeds have been reported in fast-growing seagrasses such as *Ruppia maritima*, *Halophila* spp., and *Zostera marina* which produce dormant seeds with hard pericarps (Seeliger et al. 1984; Bird and Jewett-Smith 1994; Bird et al. 1998). The hard seed coat of these species facilitates disinfection by protecting embryos from chemical damage.

In contrast, *P. oceanica* produces soft and permeable non-dormant seeds that germinate internally while still enclosed in the fruit. As the fruits mature, they dehisce spontaneously

and float due to their positive buoyancy, allowing the germinated seeds to accumulate along the shoreline (Guerero-Meseguer et al. 2018). This floating phase limits the possibility of disinfecting intact seeds, as most beach-cast fruits are already open and their seeds are exposed to environmental contamination. While disinfection of non-dehiscent fruits collected directly from the meadows has proven effective (Balestri et al. 1998), this method is logistically complex and may interfere with natural recruitment process. Moreover, non-dehiscent fruits represent a small portion of beach-cast fruits and can only be collected on the shoreline during a narrow temporal window shortly after release (Diaz-Almela et al. 2006). In this context, mature seeds from dehiscent fruits offer a more abundant and accessible source of material, often accumulating on the seashore over one or two months of the late spring. Developing a reliable disinfection protocol for these dehiscent beach-cast fruits and seeds would therefore provide a sustainable and scalable source of explants for micropropagation. Furthermore, since seeds can be maintained in aquaria for up to eight months after germination (Balestri et al. 2009), evaluating disinfection treatments for seedlings at different developmental stages could further extend the availability of viable material for micropropagation.

A wide range of disinfectants has been tested in plant micropropagation, with effectiveness depending on the species, tissue type, and treatment parameters. Sodium hypochlorite (NaClO) is the most commonly used disinfectant in seagrass tissue culture due to its broad-spectrum antimicrobial activity, low cost, and ease of use. Its effectiveness has been widely demonstrated across various species, although optimal concentration and exposure time must be carefully adjusted to minimize phytotoxic effects on delicate tissues. Balestri et al. (1998) used a concentration of 1.5% for 30 min to disinfect non-dehiscent fruits of *P. oceanica* collected from natural meadows, obtaining between 62% and 89% uncontaminated cultures. However, the percentage was significantly lower in fruits that were surface-sterilized 10 days after collection. The use of Povidone-iodine (PVP-I), commercially known as Betadine[®], has also been widely employed as an effective disinfectant in various marine plant tissue culture studies due to its broad-spectrum antimicrobial properties and relatively low phytotoxicity when used at appropriate concentrations and exposure times. Concentrations between 0.01% and 10%, applied for 30 s to a few minutes has been successfully used to reduce microbial contamination in seagrass seeds (*Halophila decipiens*) (Bird et al. 1998), *Zostera* roots (Fuggle et al. 2023), and macroalgal explants (*Enteromorpha*, *Gracilaria*, *Kappaphycus*) (Young Cho et al. 2001; Saminathan et al. 2015; Ali et al. 2020), without causing significant tissue damage. Antifungal agents like Benlate, Benomyl[®], and Captan[®], have been

used for disinfecting seagrass material for in vitro purposes; however, their effectiveness varied greatly depending on the compound's composition and the species being treated (Loquès et al. 1990; Koch and Durako 1991; Bird et al. 1993). Methyl thiophanate (TM), a broad-spectrum fungicide used in terrestrial tissue culture, remains underexplored for marine macrophytes and could be particularly promising for explants derived from field material, such as *P. oceanica* seeds obtained from beach-cast fruits.

The main objective of this study is to develop a reliable disinfection protocol for seeds and seedlings of *P. oceanica* derived from beach-cast fruits, providing a foundational step toward future micropropagation. To do that, three disinfection sets of experiments were applied to three different planting materials: (1) newly released seeds from beach-cast non-dehiscent fruits, (2) 2-month-old and (3) 6-month-old seedlings from dehiscent fruits. Finally, the most effective combination of plant material and disinfection treatment is proposed as a reproducible protocol for future *P. oceanica* micropropagation initiatives.

Methods

Non-dehiscent and dehiscent fruits were manually collected from beach wrack on several beaches of Majorca (Balearic Islands, Spain) between May and June 2023. Non-dehiscent fruits were maintained in aerated aquaria with artificial seawater (salinity 36 practical salinity units (psu)) inside an environmental chamber (Walk-in, Dycometal, Barcelona, Spain) set to 21 °C with 12:12 h light: dark photoperiod for one to two days until disinfection treatments with newly released seeds were applied.

Seeds from dehiscent fruits were manually separated from their pericarp and germinated under non-axenic conditions in aerated aquaria filled with artificial seawater (Pro-Reef Sea Salt, Tropic Marin; Supplementary Table 1). They were maintained under the same temperature and light conditions described above until their use in the disinfection experiments with 2-month-old and 6-month-old seedlings. No substrate was used in the aquaria; the germinated seedlings drifted freely over the glass bottom in aerated and filtered seawater until they were used in the experiments. Multiple aquaria were used to ensure that light was evenly distributed among all the cultivated seedlings. Newly released seeds did not yet have roots or leaves, 2-month-old seedlings already had primary and secondary roots formed, as well as leaves, and 6-month-old seedlings had considerably larger leaves and roots (Table 1).

Three sets of experiments, each involving several disinfection treatments, were conducted by using newly released seeds and 2-month-old and 6-month-old seedlings of *P.*

oceanica (Table 1). Newly released seeds were extracted from disinfected non-dehiscent fruits, while disinfection experiments with seedlings were conducted on seedlings from seeds that were germinated in aquaria, and which were extracted from dehiscent fruits (Table 1).

Disinfection treatments were applied by soaking *P. oceanica* material in different compound solutions for varying concentrations and durations (Table 1). Three disinfection compounds were used in the set of experiments: sodium hypochlorite (NaClO), povidone-iodine (PVP-I), and the antifungal thiophanate-methyl (TM). The concentration and soaking time used for each disinfectant compound were selected based on scientific published evidence on disinfection protocols in seagrasses and other marine macrophytes, with adjustments made to minimize tissue damage while ensuring effective microbial control.

Once the disinfection treatments were applied, axenic cultures were kept in an environmental chamber at the same seawater temperature, light irradiance ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$), and photoperiod conditions as described above. Axenic culture survival (defined as the percentage of live replicates without visible contamination) was monitored at weeks 1, 2, 4, 8, 12, 24, 40, and 48 to compare treatment effectiveness over time. Cultures were considered axenic when no visible signs of microbial contamination (e.g., turbidity, fungal hyphae, bacterial colonies or biofilms) were observed in the culture medium or on plant tissues for the duration of the experiment. The culture media of axenic cultures were renewed under axenic conditions every 8 weeks.

Disinfection treatments on newly released seeds

A total of 180 non-dehiscent fruits were employed in the disinfection treatments targeting newly released seeds. Half of the fruits ($N=90$) were previously submerged in 3.5% NaClO diluted in autoclaved artificial seawater (ASWa; salinity 36 psu) for 15 min, while the other half were kept in ASWa for 15 min (Table 1). Afterward, all fruits were rinsed three times in ASWa, and during the final rinse, they were soaked for 10 min in ASWa. Given the collection context of the non-dehiscent fruits (shoreline wrack), a higher NaClO concentration than in Balestri et al. (1998) was used, and the exposure time was reduced to mitigate potential seed damage.

Fruit pericarps were carefully opened under axenic conditions in a laminar flow cabinet using sterilized scalpels, forceps, and Petri dishes to obtain newly released seeds for the experiment. Subsequently, newly released seeds were exposed to the corresponding disinfection treatments ($n=15$) inside a laminar flow cabinet. These treatments involved exposing the newly released seeds, whose fruit pericarps had previously been treated with ASWa and NaClO, to two

Table 1 Plant materials and disinfection treatments used in this study. Treatments were applied to newly released seeds from non-dehiscent fruits, as well as to 2-month-old and 6-month-old seedlings derived from dehiscent fruits of *Posidonia oceanica*. Disinfection compounds included sodium hypochlorite (NaClO), povidone-iodine (PVP-I), and thiophanate-methyl (TM), applied at varying concentrations and durations as indicated. In the case of newly released seeds, NaClO was applied to the fruits, whereas for seedlings, NaClO was applied directly. Treatments soaked only in ASWa were considered controls. All resulting treatments were cultured in ASWa, both with and without the antibiotic Timentin (ticarcillin+clavulanic acid). ASWa, autoclaved artificial seawater; 3×NaClO, triple sodium hypochlorite pretreatment applied to 6-month-old seedlings

Plant material	Compounds used				Resulting disinfection treatment
	NaClO (% _v , min)	PVP-I (% _v , min)	TM (mg L ⁻¹)	Tim (mg L ⁻¹)	
Newly released seeds from non-dehiscent fruits	—	—	—	—	Control
	—	—	—	+	Control + Tim
	—	1, 10	—	—	1PVP-I+ASWa
	—	1, 10	—	+	1PVP-I+Tim
	—	3, 10	—	—	3PVP-I+ASWa
	—	3, 10	—	+	3PVP-I+Tim
	3.5, 15	—	—	—	NaClO+ASWa
	3.5, 15	—	—	+	NaClO+Tim
	3.5, 15	1, 10	—	—	NaClO+1PVP-I+ASWa
	3.5, 15	1, 10	—	+	NaClO+1PVP-I+Tim
	3.5, 15	3, 10	—	—	NaClO+3PVP-I+ASWa
	3.5, 15	3, 10	—	+	NaClO+3PVP-I+Tim
2-month-old seedlings 	—	—	—	—	Control
	—	—	—	+	Control + Tim
	0.01, 5	—	—	—	0.01NaClO+ASWa
	0.01, 5	—	—	+	0.01NaClO+Tim
	0.1, 5	—	—	—	0.1NaClO+ASWa
	0.1, 5	—	—	+	0.1NaClO+Tim
	0.5, 5	—	—	—	0.5NaClO+ASWa
	0.5, 5	—	—	+	0.5NaClO+Tim
	1, 5	—	—	—	1NaClO+ASWa
	1, 5	—	—	+	1NaClO+Tim
	—	1, 5	—	—	1-5PVP-I+ASWa
	—	1, 5	—	+	1-5PVP-I+Tim
	—	1, 10	—	—	1-10PVP-I+ASWa
	—	1, 10	—	+	1-10PVP-I+Tim
	—	3, 5	—	—	3-5PVP-I+ASWa
	—	3, 5	—	+	3-5PVP-I+Tim
	—	3, 10	—	—	3-10PVP-I+ASWa
	—	3, 10	—	+	3-10PVP-I+Tim
	—	1, 5	0.5, 5	—	1PVP-I+0.5TM+ASWa
	—	1, 5	0.5, 5	+	1PVP-I+0.5TM+Tim
	—	1, 5	1, 5	—	1PVP-I+1TM+ASWa
	—	1, 5	1, 5	+	1PVP-I+1TM+Tim
	—	1, 5	2, 5	—	1PVP-I+2TM+ASWa
	—	1, 5	2, 5	+	1PVP-I+2TM+Tim
6-month-old seedlings	—	—	—	—	Control
	—	—	—	+	Control + Tim
	0.5, 5	—	—	—	0.5NaClO+ASWa
	1, 5	—	—	—	1NaClO+ASWa
	—	1, 5	0.5, 5	—	1PVP-I+0.5TM+ASWa
	—	1, 5	1, 5	—	1PVP-I+1TM+ASWa

Table 1 (continued)

Plant material	Compounds used				Resulting disinfection treatment
	NaClO (% , min)	PVP-I (% , min)	TM (mg L ⁻¹)	Tim (200 mg L ⁻¹)	
	–	1, 5	2, 5	–	1PVP-I+2TM+ASWa
	3×0.01, 5	–	–	–	3×NaClO+ASWa
	3×0.01, 5	–	–	+	3×NaClO+Tim
	3×0.01, 5	1, 5	–	–	3×NaClO+1PVP-I+ASWa
	3×0.01, 5	1, 5	–	+	3×NaClO+1PVP-I+Tim
	3×0.01, 5	1, 5	0.5, 5	–	3×NaClO+1PVP-I+0.5TM+ASWa
	3×0.01, 5	1, 5	0.5, 5	+	3×NaClO+1PVP-I+0.5TM+Tim
	3×0.01, 5	1, 5	1, 5	–	3×NaClO+1PVP-I+1TM+ASWa
	3×0.01, 5	1, 5	1, 5	+	3×NaClO+1PVP-I+1TM+Tim
	3×0.01, 5	1, 5	2, 5	–	3×NaClO+1PVP-I+2TM+ASWa
	3×0.01, 5	1, 5	2, 5	+	3×NaClO+1PVP-I+2TM+Tim

concentrations of PVP-I for 10 min (Table 1). To assess contamination effects, a Control treatment was included in which non-dehiscent fruits and the resulting newly released seeds were immersed in ASWa alone for 15 and 5 min, respectively. Following each disinfection treatment, the newly released seeds were rinsed three times in ASWa.

After treatment, each seed was individually placed in capped test tubes (25 × 250 mm of diameter) containing either ASWa or ASWa supplemented with 200 mg L⁻¹ of Timentin (Tim; ticarcillin + clavulanic acid) as culture medium, to evaluate the effect of this antibiotic on the survival of axenic cultures (Table 1). Axenic culture survival was monitored over the corresponding weeks and additionally, the germination success was evaluated. If the seed showed no signs of germination within four weeks, the seed was considered inert. Then, the percentage of germination was calculated for each treatment after four weeks of culture.

Disinfection treatments on 2-month-old seedlings

Two-month-old seedlings ($n=10$) (cultured in aquaria under the controlled conditions described above) were exposed to disinfection treatments under axenic conditions inside a laminar flow cabinet. Four concentrations each of NaClO and PVP-I, follow by three concentrations of TM, were tested with a 5-minutes soak time in the disinfection treatments targeting 2-month-old seedlings (Table 1).

The seedlings were rinsed three times in ASWa after the expose to a disinfectant. In treatments with more than one disinfectant agent, the seedlings were rinsed three times in ASWa before being exposed to the second chemical. To assess contamination effects, a Control treatment was included in which the 2-month-old seedlings were immersed ASWa alone for 5 min.

Following their respective treatments, each seedling was individually placed in test tubes (25 × 250 mm) containing ASWa, with or without the addition of 200 mg L⁻¹ Tim, as the culture medium, to evaluate the effect of this antibiotic on the axenic culture survival (Table 1).

Disinfection treatments on 6-month-old seedlings

Seedlings were grown in aquaria filled with artificial seawater for six months under the same controlled conditions as the 2-month-old seedlings. Prior to the experiments, brown leaves were removed from the 6-month-old seedlings, and the green leaves were lightly trimmed to eliminate any brown apical parts, ensuring that only live tissue remained in the explants. This also standardized leaf size across all the seedlings treated (5–7 cm in length; Fig. 1). Leaf trimming was not expected to affect seedling development, as previous studies simulating grazing in *P. oceanica* have shown that even more extensive leaf removal does not compromise growth rates, despite reducing biomass, and may even trigger compensatory responses (Cebrián et al. 1998; Vergés et al. 2008; Buñuel et al. 2023). Subsequently, seedlings were subjected to the disinfection treatments ($n=20$) that had yielded the highest axenic culture survival in previous trials with 2-month-old seedlings. An additional set of trials applied the same treatments ($n=20$), preceded by a pre-treatment consisting of three consecutive 5-minute immersions in 0.01% NaClO (3× NaClO), with 10-minute rinses in ASWa between each immersion (Table 1). To assess contamination effects, a Control treatment was included in which the 6-month-old seedlings were immersed ASWa alone for 5 min.

Following their respective disinfection treatments, seedlings were individually placed in test tubes (25 × 250 mm of

Fig. 1 Axenic (A) and contaminated (B) cultures of newly released seeds of *Posidonia oceanica* after one week of culture, and axenic cultures from newly released seeds after 48 weeks of culture (C)



diameter) containing ASWa culture medium, either supplemented with 200 mg L^{-1} of the Tim or left without it, to evaluate the effect of this antibiotic in the survival of axenic cultures (Table 1).

Statistical analyses

Differences in axenic culture survival across disinfection treatments and concentrations of each disinfectant were analysed using Cox Proportional Hazards Models. Analyses were conducted independently for each developmental stage. This model is commonly employed in survival analysis to assess the association between predictor variables and the time until an event of interest occurs, in our case, the axenic culture survival. The variables used in the Cox Proportional Hazards Model include “Time”, which is a continuous variable representing the week at which the axenic culture survival was checked; and “Survival”, a binary variable indicating whether survival occurred (coded as 1) or not (coded as 0) for each observation. Additionally, the disinfection treatment was included in the model as a categorical variable (factor). This allows the model to estimate how axenic culture survival differs across treatment groups, with the Control treatment serving as the reference category. In cases where no replicates from the Control treatment achieved axenic conditions by week 1, the treatment exposed to the lowest concentration and fewest disinfection compounds that showed axenic culture survival from the first week was used as the reference in statistical analyses. This was the case for 2-month-old and 6-month-old seedlings, where the 0.5NaClO –ASW treatment was used as the reference for statistical comparisons. To further assess the specific effects of individual disinfectant components (NaClO, PVP-I, and Tim), we conducted an additional Cox Proportional Hazards Model in which the concentration of each compound was included as an independent categorical variable (with 0 representing absence).

Differences in germination among treatments were first assessed using generalized linear models (GLM) with a

binomial error distribution and a logit link function, where the number of germinated seeds out of the total tested was used as the response. Bias-reduced estimation (Firth-type penalization) was applied using the *brglm2* package to account for complete or quasi-complete separation. In addition to the full-treatment analysis, we separately evaluated the effects of individual disinfectant components (NaClO, PVP-I, and Tim). For each disinfectant component, GLMs with the same structure (binomial error and logit link) were fitted independently.

All values are reported as mean $\pm$ standard error (SE), unless otherwise indicated. Statistical analyses were conducted with a significance level of $\alpha=0.05$, using R version 4.3.2 (R Core Team 2023) within the RStudio environment (version 2023.12.1+402, Posit Software PBC 2024).

Results

Axenic cultures from newly released seeds

The seeds that remained axenic throughout the experiment maintained a clear and transparent medium (Fig. 1A). In contrast, some of the newly released seeds showed signs of contamination during the first week, resulting in a dense and whitish culture medium (Fig. 1B).

Binomial GLM analyses revealed significant differences in germination success among treatments, with 1PVP-I+Tim ($p=0.021$), 3PVP-I+ASWa ($p=0.006$), and NaClO+ASWa ($p=0.021$) showing the lowest germination success (Supplementary Table 3). In separate comparisons for disinfectant components, the use of 3% PVP-I significantly reduced germination success ($p=0.020$) compared to 1% PVP-I and no treatment, whereas NaClO ($p=0.185$) and Tim ($p=0.658$) had no significant impact.

Seed germination percentages per treatment after four weeks of culture ranged from $26.67 \pm 23.66\%$ to $93.33 \pm 6.47\%$ (mean $\pm$ 95% confidence interval; Supplementary Table 2), with an overall average of $48.61 \pm 7.23\%$

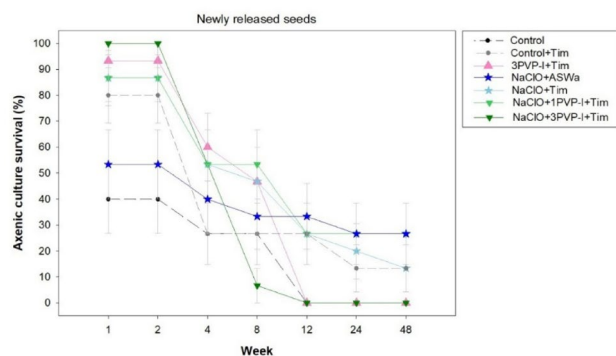


Fig. 2 Axenic culture survival (%) over time (mean±SE) for *Posidonia oceanica* newly released seeds ($n=15$) subjected to different disinfection treatments. Only treatments showing positive and significant coefficients in the Cox Proportional Hazards Model compared with the Control treatment are shown

Table 2 Summary of Cox proportional hazards model results evaluating the effect of disinfection treatments on axenic culture survival in newly released seeds ($n=15$), relative to the control treatment. Significant results ($p<0.05$) are shown in bold

Treatment	Coefficient	Lower confidence interval	Upper confidence interval	p -value
NaClO+1PVP-I+Tim	1.014	0.499	1.528	0.001
NaClO+Tim	0.953	0.435	1.472	0.001
3PVP-I+Tim	0.824	0.295	1.352	0.002
Control+Tim	0.719	0.182	1.255	0.009
NaClO+ASWa	0.712	0.175	1.249	0.009
NaClO+3PVP-I+Tim	0.699	0.160	1.238	0.011
1PVP-I+Tim	0.311	-0.267	0.889	0.292
NaClO+1PVP-I+ASWa	0.271	-0.311	0.854	0.361
1PVP-I+ASWa	-0.104	-0.741	0.533	0.748
NaClO+3PVP-I+ASWa	-0.519	-1.234	0.197	0.155
3PVP-I+ASWa	-16.70	-1847.6	1814.2	0.986

(mean±95% confidence interval). Since all newly released seeds from the 3PVP-I+ASWa treatment were contaminated during the first week of culture, germination success could not be assessed for this treatment. Nevertheless, the seeds that did germinate in the other treatments were able to develop leaves and roots and continued growing for up to 48 weeks in some cases (Figs. 1C and 2, Supplementary Table 3).

Cox Proportional Hazards regression analyses revealed significantly higher hazard coefficients compared to the control treatment in six out of the eleven disinfection treatments applied to newly released seeds (Table 2; Fig. 2). Among these, the treatments NaClO+1PVP-I+Tim and NaClO+ASWa achieved the highest percentages of axenic cultures after 48 weeks, both reaching a survival rate of $26.67\pm 11.82\%$ (Fig. 2A; Table 2, Supplementary Table 3). The Control+Tim and NaClO+Tim treatments attained $13.30\pm 9.08\%$ axenic cultures after 48 weeks

Table 3 Summary of Cox proportional hazards model results for 2-month-old seedlings ($n=10$), assessing the effect of disinfection treatments on axenic culture survival, using 0.5NaClO–ASWa as the reference treatment. Significant results are highlighted in bold

Treatment	Coefficient	Lower confidence interval	Upper confidence interval	p -value
1PVP-I+1TM+ASWa	-0.141	-0.504	0.222	0.446
1NaClO+ASWa	-0.184	-0.550	0.183	0.326
1PVP-I+0.5TM+ASWa	-0.226	-0.597	0.144	0.231
1PVP-I+2TM+ASWa	-0.252	-0.624	0.121	0.186
1PVP-I+2TM+Tim	-0.296	-0.673	0.081	0.124
0.5NaClO+Tim	-0.345	-0.727	0.037	0.076
1PVP-I+1TM+Tim	-0.498	-0.896	-0.100	0.014
1NaClO+Tim	-0.583	-0.991	-0.176	0.005

(Fig. 2, Supplementary Table 3). However, despite initially high proportions of axenic cultures, all replicates in the 3PVP-I+Tim and NaClO+3PVP-I+Tim treatments became contaminated by weeks 12 and 8, respectively (Fig. 2, Supplementary Table 3).

Cox Proportional Hazards regression analyses conducted separately for NaClO, PVP-I, and Tim revealed significant effects across the concentrations tested. A 10-minute soak in 3.5% NaClO significantly improved axenic culture survival of non-dehiscent fruits ($p<0.001$, coefficient=0.403). In contrast, the 3% PVP-I concentration was associated with a significantly negative effect on axenic culture survival ($p<0.001$, coefficient = -0.466). Finally, the inclusion of Tim in the culture medium had a significant positive effect ($p<0.001$, coefficient=0.811).

Axenic cultures from 2-month-old seedlings

Of the disinfection treatments tested on 2-month-old seedlings, fifteen showed signs of contamination and a survival probability of 0% by the first week of culture (Supplementary Table 4). In all these cases, the presence of small crustaceans (copepods) was observed between the rhizome and leaves of the seedlings. As a result, these treatments were not included in the analysis of survival over time. The 0.5NaClO+ASWa treatment, which showed no signs of contamination and no presence of copepods, was selected as the reference for comparing axenic culture survival over time in the Cox Proportional Hazards Model regressions.

Cox Proportional Hazards Model regressions identified significantly lower axenic culture survival in the treatments 1NaClO+Tim and 1PVP-I+1TM+Tim compared with the 0.5NaClO+ASWa treatment (Table 3, Supplementary Table 4). In contrast, the remaining treatments tested in the Cox Proportional Hazards Model regressions exhibited axenic culture survival percentages comparable to those observed with the 0.5NaClO+ASWa treatment, which showed the

highest survival after 40 weeks (Figs. 3A and 4B; Table 3, Supplementary Table 4).

During the initial weeks of culture, the leaves of the 2-month-old seedlings treated with NaClO exhibited a loss of green coloration and the appearance of brownish hues, with more pronounced effects at higher dosages (Fig. 4A). However, starting from the first month, leaf growth resumed, and a green coloration was gradually recovered, continuing through the 40 weeks of culture (Fig. 4B).

Cox Proportional Hazards regression analyses conducted separately for the compounds NaClO, PVP-I, TM and Tim, also revealed significant coefficients across the concentrations utilized. The use of 0.5% ($p < 0.001$, coefficient = 1.344) and 1% of NaClO ($p < 0.001$, coefficient = 1.344) showed significantly higher axenic culture survival percentage than the untreated seedlings. Regarding the use of PVP-I, only the application of a concentration of 1% for 5 min showed significantly positive results on axenic culture survival ($p < 0.001$, coefficients = 0.452). Axenic culture survival of seedlings treated with 0.5 mg L⁻¹ ($p < 0.001$, coefficient = 0.869), 1 mg L⁻¹ ($p < 0.001$, coefficient = 1.534) and 2 mg L⁻¹ of TM were higher than in untreated seedlings. In contrast, the use of Tim exhibited significantly negative

results ($p < 0.001$, coefficient = -0.478) compared to the untreated seedlings.

Axenic cultures from 6-month-old seedlings

None of the disinfection treatments tested in 6-month-old seedlings had a percentage of axenic culture survival exceeding 15% by the 12 weeks of culture, prompting the termination of the experiment in this stage (Fig. 3B, Supplementary Table 5). In the first week of culture, two of the tested treatments, Control and Control+Tim, showed contamination of small crustaceans (copepods) in the culture media, so these treatments were not considered for statistical analysis. Thus, the treatment that was least exposed to disinfectants, 0.5NaClO+ASWa, was used as the reference treatment for the Cox Proportional Hazards Model regressions. The rest of the treatments, except for 0.5NaClO+ASWa and 1NaClO+ASWa, also showed contamination by copepods in some of the replicates to a greater or lesser extent. These replicates were considered contaminated and eliminated from the experiment in the same way as those that showed a contaminated culture media.

The Cox Proportional Hazards Models regressions showed significant negative coefficients in the treatments 1PVP-I+0.5TM+ASWa, 1PVP-I+1TM+ASWa, 1PVP-I+2TM+ASWa, 3×NaClO+ASWa, and 3×NaClO+1PVP-I+Tim compared with the 0.5NaClO+ASWa treatment (Table 4). These treatments resulted in lower axenic culture survival percentages than the other treatments (Supplementary Table 5). The treatment that achieved the highest axenic culture survival at 12 weeks of culture was 3×NaClO+1PVP-I+0.5TM+ASWa (Fig. 3B; Table 4, Supplementary Table 5).

Regarding the Cox Proportional Hazards Model regressions separately tested for NaClO, PVP-I, TM and Tim, significant coefficients were also observed in the treated seedlings compared to the untreated ones. All concentrations of NaClO used, including 1% ($p < 0.001$, coefficient = 1.522), 0.5% ($p < 0.001$, coefficient = 1.551), and the application as a pretreatment of 0.01% three times ($p < 0.001$, coefficient = 1.199), showed significant positive effects on axenic culture survival. In contrast, the use of PVP-I did not show a significant effect ($p = 0.400$, coefficient = 0.086). In TM, only the concentration of 2 mg L⁻¹ caused significant positive effects on axenic culture survival ($p = 0.037$, coefficient = 0.261). Finally, the use of Tim in the culture media also had significant positive effects on axenic culture survival ($p < 0.001$, coefficient = 0.370).

Similarly, to what occurred in 2-month-old seedlings, the 6-month-old seedlings treated with NaClO exhibited whitening of their leaves, which intensified with the concentration of this compound (Fig. 5). This whitening persisted

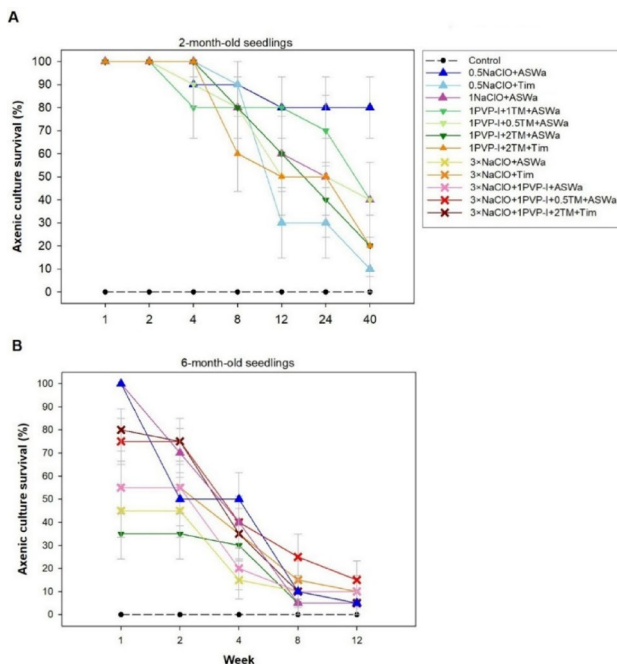


Fig. 3 Axenic culture survival (%) over time (mean ± SE) for 2-month-old (A) ($n = 10$) and 6-month-old (B) ($n = 20$) seedlings of *Posidonia oceanica* subjected to different disinfection treatments. Only treatments with surviving seedlings at the end of the experiment are shown. Control: seedlings were only exposed to autoclaved artificial seawater (ASWa). NaClO: sodium hypochlorite. PVP-I: povidone-iodine. TM: thiophanate methyl. 3×NaClO, triple sodium hypochlorite pretreatment applied in 6-month-old seedlings. ASWa: autoclaved artificial seawater as culture medium. Tim: ASWa supplemented with 200 mg L⁻¹ of the antibiotic Timentin as culture medium

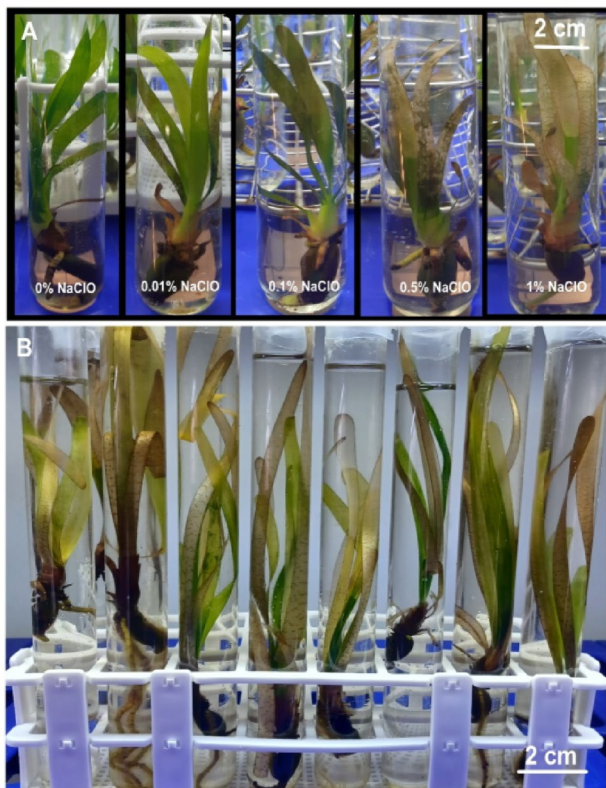


Fig. 4 Effect of sodium hypochlorite (NaClO) on the leaf appearance of 2-month-old *Posidonia oceanica* seedlings. **(A)** Seedlings after one week of culture. **(B)** Axenic cultures treated with 0.5% NaClO for 5 min, observed after 40 weeks of culture

throughout the 12 weeks of axenic culture; however, unlike the 2-month-old seedlings, the treated seedlings eventually died.

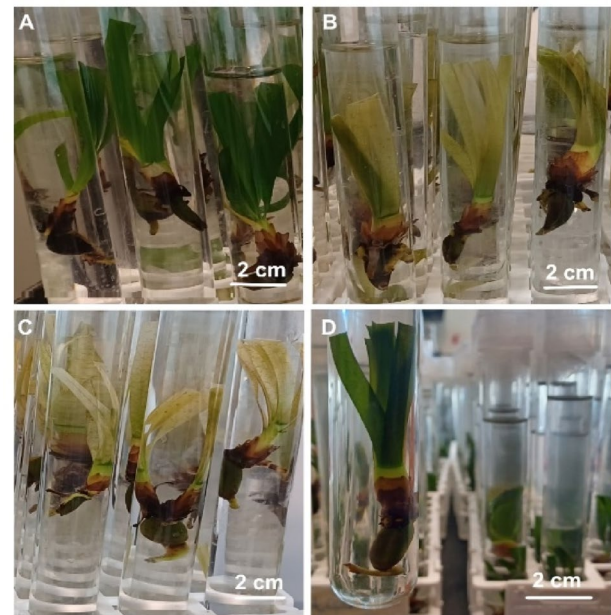


Fig. 5 Effect of NaClO on 6-month-old seedling leaves. **(A)** Non-treated 6-month-old seedlings; **(B)** 6-month-old seedlings treated with 0.5%; **(C)** 6-month-old seedlings treated with 1% of NaClO; and **(D)** Axenic aspect of 6-month-old seedlings treated with a pretreatment of 0.01% of NaClO for 5 min, repeated three times

Discussion

The findings of this study provide the first robust evidence that axenic cultures of *Posidonia oceanica* seedlings derived from beach-cast fruits can be successfully maintained for up to 48 weeks. Seedling survival under axenic conditions was influenced by multiple factors, including seedling age, the type and concentration of disinfection compounds used, and the duration of exposure. In the following sections, we discuss the main findings in relation to the disinfectant compounds tested and examine how seedling age modulated

Table 4 Summary of Cox proportional hazards model results obtained for 6-month-old seedlings ($n=20$) evaluating the effect of disinfection treatments on axenic culture survival, using 0.5NaClOASWa as the reference treatment. Significant results are highlighted in bold

Treatment	Coefficient	Lower confidence interval	Upper confidence interval	<i>p</i> -value
3×NaClO+1PVP-I+0.5TM+ASWa	0.069	−0.347	0.484	0.746
1NaClO+ASWa	0.029	−0.391	0.449	0.892
3×NaClO+1PVP-I+2TM+Tim	−0.049	−0.477	0.379	0.823
3×NaClO+1PVP-I+1TM+Tim	−0.217	−0.663	0.229	0.341
3×NaClO+Tim	−0.256	−0.705	0.194	0.265
3×NaClO+1PVP-I+2TM+ASWa	−0.343	−0.804	0.119	0.146
3×NaClO+1PVP-I+ASWa	−0.386	−0.852	0.080	0.105
3×NaClO+1PVP-I+0.5TM+Tim	−0.387	−0.853	0.080	0.104
3×NaClO+1PVP-I+1TM+ASWa	−0.416	−0.887	0.055	0.084
3×NaClO+ASWa	−0.620	−1.120	−0.121	0.015
1PVP-I+2TM+ASWa	−0.713	−1.226	−0.199	0.007
3×NaClO+1PVP-I+Tim	−0.754	−1.276	−0.232	0.005
1PVP-I+0.5TM+ASWa	−3.147	−4.565	−1.729	0.001
1PVP-I+1TM+ASWa	−3.842	−5.825	−1.859	0.001

their effectiveness, situating our results within the broader context of previous studies on seagrasses.

Effectiveness of NaClO as a disinfectant for *Posidonia oceanica* seedlings

The application of NaClO consistently produced positive disinfection outcomes across all tested seedling ages. When applied to non-dehiscent fruits, NaClO increased the axenic culture survival percentage by 12% compared to untreated fruits after 48 weeks of culture. Previous studies on non-dehiscent fruits of *Posidonia* spp. used lower NaClO concentrations (between 1% and 1.5%) than those applied in our study (Balestri et al. 1998; Henry 1998). However, the higher concentration used here (3.5%) did not negatively impact seed germination, as similar germination rates were observed in untreated fruits (43%) and treated ones (53%). Moreover, the germination rates recorded were within the range previously reported for *P. oceanica* under axenic conditions by Balestri et al. (1998), which varied between 30% and 96%.

For 2-month-old seedlings, a 5-minute soak in 0.5% NaClO yielded the best disinfection results, with 80% of cultures remaining viable and axenic after 40 weeks. Although higher concentrations, such as 1% NaClO, also produced axenic conditions, they reduced seedling survival to approximately 40%, likely due to increased phytotoxicity. Thus, 0.5% NaClO offered double the survival rate compared to 1%, indicating a critical balance between disinfection efficacy and tissue tolerance. Similarly, in 6-month-old seedlings, NaClO treatments improved axenic culture survival by up to 20% during the first 12 weeks. The lower survival observed in this older group compared to the 2-month-old seedlings may be attributed to the dissection procedure required prior to disinfection. Leaf trimming likely increased the penetration of NaClO into plant tissues, negatively affecting the seedlings' regeneration capacity. In contrast, younger seedlings with smaller leaves did not require dissection and showed healthy recovery within a few weeks after treatment. These results indicate that NaClO concentrations between 0.1% and 1% can effectively disinfect *P. oceanica* seedlings. However, disinfection protocols should be carefully optimized when tissue dissection is involved, in order to minimize phytotoxic effects and improve survival in axenic culture.

In colonizing seagrasses of the genus *Halophila*, NaClO has been widely used at concentrations ranging from 0.5% to 6%, achieving nearly complete microbial decontamination (approximately 100%) of explants (Jewett-Smith and Mcmillan 1990; Bird et al. 1998). In *Ruppia maritima*, use of 0.525% NaClO resulted in 25–40% axenic culture success (Bird et al. 1996). Recent studies have applied NaClO

for axenic experimentation with seeds of the seagrass *Z. marina*, obtaining satisfactory results with both seed germination and the development of the resulting seedlings (Li et al. 2025; Pieraccini et al. 2025). Considering that these species produce dormant seeds with harder, less permeable seed coats compared to the foam-like seed coat of *P. oceanica*, the approximately 80% axenic culture survival maintained for 40 weeks using 0.5% NaClO in our study indicates a promising level of disinfection success. This success is comparable, or even superior, to that reported for other seagrass species, underscoring the value of NaClO as a standard treatment in axenic culture protocols and providing a solid foundation for future micropropagation research.

The use of PVP-I to obtain axenic cultures of *P. oceanica* seedlings

In this study, the application of 3% PVP-I for 5 min to newly released *P. oceanica* seeds reduced germination rates by approximately 20% compared to seeds treated with 1% PVP-I and untreated controls (33% vs. 55% and 56%, respectively). Moreover, PVP-I treatment did not support seedling development and survival under axenic conditions, except at the 1% concentration for 5 min when combined with NaClO and Timentin. For 2-month-old seedlings, comparable results were observed: seedlings treated with 1% PVP-I for 5 min showed the highest axenic survival, particularly when combined with TM. In contrast, extending the exposure time to 10 min or increasing the concentration to 3%, with or without additional compounds, resulted in complete mortality of all replicates. These results indicate that concentrations above 1% and exposure times longer than 5 min are detrimental to seedling viability.

PVP-I treatments had no significant disinfectant effect on 6-month-old seedlings, either when used alone or in combination with TM. Therefore, PVP-I appears to be an effective disinfectant for *P. oceanica* seedlings up to two months of age when applied at concentrations of 1% or lower and with exposure times under 5 min, especially when used in conjunction with complementary compounds that enhance disinfection efficacy.

These findings align with previous studies in other seagrass species. For instance, a low concentration of PVP-I (0.01%) applied for 1 min effectively disinfected *Halophila decipiens* seeds prior to germination under sterile conditions, resulting in nearly 100% axenic cultures (Bird et al. 1998). Conversely, higher PVP-I concentrations (10%) were shown to disrupt root-associated microorganisms in *Zostera* spp., reducing bacterial and archaeal abundance by approximately 85–95% compared to untreated controls, without directly harming the host plant (Fuggle et al. 2023). This difference may be explained by anatomical factors: roots

possess more robust, lignified tissues, whereas the seedlings in our experiment contained softer, more permeable tissues such as leaves and seeds, which could be more susceptible to damage, potentially increasing mortality or contamination rates. Therefore, the intermediate concentrations used in this study reflect a compromise between achieving effective disinfection and maintaining seedling viability.

Potential of TM as a disinfection agent for *P. oceanica* seedlings

This study presents the first reported application of the antifungal compound TM for disinfecting *P. oceanica* seedlings. In 2-month-old seedlings, axenic culture survival increased with TM concentration, reaching the highest success (70% axenic cultures after 40 weeks) when treated with 2 mg L⁻¹ TM for 5 min, combined with a 5-minute soak in 1% PVP-I. This combination also showed promising results for 6-month-old seedlings, with 31% axenic cultures achieved after 12 weeks of culture.

The use of antifungal agents in seagrass disinfection protocols has been previously explored, though results have varied depending on the species, tissue type, and disinfectant used. For example, Loquès et al. (1990) successfully used Benlate® (6 g L⁻¹, 5 min) to disinfect seeds, leaf fragments, and macromeristems of *P. oceanica*. Although the results were positive, especially when combined with other agents like calcium hypochlorite and ethanol, completely axenic cultures were not obtained. Koch and Durako (1991) applied Captan® (2.5 g L⁻¹) in combination with NaClO and antibiotics to disinfect *Ruppia maritima* rhizomes, achieving 51% axenic cultures; however, contamination by aerophilic fungi persisted, indicating limited effectiveness. Similarly, Bird et al. (1993) used a combined antibiotic and fungicide solution containing 1 g L⁻¹ Benomyl® following treatment with NaClO and sodium lauryl sulfate, but reported only 40% contamination-free cultures.

In our study, the effectiveness of TM appears to result primarily from its toxicity to small crustaceans, such as copepods, that often reside in the leaf bases or rhizome crevices of seedlings, without causing visible damage to plant tissues. While TM may not be essential for disinfecting seedlings germinated from freshly collected beach-cast fruits, where NaClO alone proved sufficient, it may offer added benefits for older seedlings grown under non-axenic aquarium conditions, particularly beyond two months of age. These findings suggest that TM has potential as a complementary disinfection agent during specific developmental stages. Future studies should evaluate alternative fungicides or optimized treatment combinations to further improve axenic culture survival, especially in older or aquarium-acclimated *P. oceanica* seedlings.

Effect of the antibiotic Tim on the disinfection of *P. oceanica* seedlings

In this study, the use of the antibiotic Tim showed no consistent or clearly beneficial effects across the different developmental stages of *P. oceanica* seedlings. While its presence in the culture medium slightly improved axenic culture survival in newly released seeds and 6-month-old seedlings, it appeared to reduce survival in 2-month-old seedlings. These findings are consistent with previous results in which antibiotic mixtures containing streptomycin, penicillin, neomycin, polymyxin, and nystatin failed to ensure long-term axenic culture maintenance in *P. oceanica* (Balestri and Cinelli 1992). Notably, the concentration of Tim applied in the present study (200 mg L⁻¹) was substantially lower than the concentrations of individual antibiotics used in Balestri and Cinelli's experiments, which ranged from approximately 220 mg L⁻¹ (nystatin at 1:100 dilution) up to 20,000 mg L⁻¹ (streptomycin at 1:10 dilution). This suggests that the concentrations of Tim tested here could be insufficient for effective bacterial suppression in *P. oceanica* seedlings, and future research should explore higher concentrations. However, the concentration used in our experiments falls within the range typically applied to other seagrass species, such as *R. maritima* (250 mg L⁻¹; Bird et al. 1993; 1996) and *Cymodocea nodosa* (150 mg L⁻¹; García-Jiménez et al. 2006), where more favourable outcomes were reported. This interspecific variation in antibiotic sensitivity and response may reflect differences in anatomy, microbial load, or microbial community composition.

Interestingly, in our study, 2-month-old seedlings cultured without antibiotics still achieved an 80% survival rate, indicating that other disinfection treatments, such as NaClO or PVP-I, may be sufficient to control bacterial contamination at this developmental stage. These results raise questions about the necessity of antibiotics in certain contexts and suggest that their inclusion should be carefully assessed based on seedling age and prior culture conditions. Future research should investigate alternative antibiotics or combinations thereof, and evaluate higher concentrations to determine whether more robust bacterial suppression can be achieved without compromising seedling viability. Such approaches may contribute to improving axenic culture maintenance and optimizing micropropagation protocols for *P. oceanica*.

Effect of seedling age on axenic culture success

In this study, the age of *P. oceanica* seedlings clearly influenced the success of axenic culture establishment. The highest percentages of axenic cultures were obtained from 2-month-old seedlings, while both newly released seeds and

6-month-old seedlings showed considerably lower success rates.

The germination rate of newly released seeds under axenic conditions averaged 49% in our experiments, which falls within the range (30–96%) reported by Balestri et al. (1998) for axenic seeds. Differences in germination rates may be influenced by seed origin and environmental conditions prior to collection. Our seeds were obtained from beach-cast fruits that had been transported by currents and waves. Such fruits could have experienced environmental stresses, including exposure to solar radiation, desiccation, or microbial colonization, which can affect seed viability. Recent studies have shown that the viability of beach-cast *P. oceanica* seeds is highly variable and depends on factors such as exposure time on the shore and environmental conditions (Sutera et al. 2024). Although we made efforts to select freshly dehiscid fruits and rapidly transport them to the laboratory aquaria, pre-collection stressors could have contributed to variability in germination and axenic culture success.

By contrast, 6-month-old seedlings showed the lowest percentage of axenic cultures following disinfection treatments. Their prolonged maintenance under non-sterile aquarium conditions likely favoured colonization by fungi, bacteria, algae, and small invertebrates, which reduced the efficacy of disinfectants. These seedlings also required trimming of senescent or damaged leaf tissue to ensure full submersion in the culture medium. This step, not needed for younger seedlings, may have increased permeability to toxic compounds during disinfection, thereby decreasing survival. Additionally, *P. oceanica* is known to accumulate pollutants in its tissues, especially in younger leaves (Richir et al. 2013), which could further reduce viability after chemical exposure.

Considering these findings, we recommend carrying out disinfection treatments shortly after seed germination, once the seedlings have developed early root and leaf structures but before they reach two months of age. Waiting too long may require more invasive handling and increase the risk of microbial colonization, including by copepods or other invertebrates, whose eggs or larvae may go unnoticed and later hatch in culture. Early-stage seedlings appear to offer the best compromise between structural robustness and manageable levels of contamination.

Conclusions

This study demonstrates, for the first time, that axenic cultures of *P. oceanica* seedlings can be successfully established from dehiscid fruits collected from beach-cast material, offering a sustainable and non-invasive alternative

to the use of fruits harvested directly from underwater meadows. This finding holds important implications for the future micropropagation and restoration of this endangered seagrass species.

Among the disinfection treatments tested, NaClO emerged as the most effective agent, achieving the highest proportion of axenic cultures across all seedling stages. The highest axenic culture survival rate, up to 80% after 40 weeks of in vitro maintenance, was obtained with 2-month-old seedlings treated with 0.5% NaClO for 5 min. This represents the highest long-term axenic survival rate reported to date for the genus *Posidonia*. The optimal disinfection window appears to be shortly after germination, once root and leaf structures are established but before microbial colonization advances or tissue damage necessitates intervention. This timing, combined with a simple and reproducible protocol, provides a strong foundation for developing large-scale propagation strategies.

While restoration efforts using *P. oceanica* seedlings have so far had limited success compared to those using mature rhizomes (Castejón-Silvo and Terrados 2021), our results represent a key methodological advance toward overcoming this limitation. Seedling micropropagation from beach-cast fruits offers several advantages, including enhanced genetic diversity, selection of phenotypes with better transplant performance, and large-scale production from non-destructive sources. Maintaining axenic seedlings for up to 40 weeks provides a valuable timeframe to test culture media and growth-promoting treatments aimed at accelerating shoot development. Future research should focus on optimizing these conditions to enhance multiplication and rooting of in vitro-propagated seedlings, thereby facilitating their application in practical restoration efforts.

Based on our findings, we recommend a two-phase approach: an initial germination and growth period in aquaria for up to two months, followed by surface disinfection using 0.5% NaClO for 5 min. This strategy, applied to seedlings from beach-cast fruits, can yield up to 80% long-term axenic cultures, providing a robust and reproducible foundation for the future development of micropropagation protocols and reducing dependence on natural *P. oceanica* populations for restoration initiatives.

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Author contributions All authors contributed significantly to the development of this research. LG-M and CLE were responsible for the conceptualization, methodology design, formal analysis, and were involved in drafting the manuscript. The experiments and data collection were performed by LG-M and MR-B. IC-S contributed to data interpretation, provided critical revisions, and assisted with manuscript editing. JT was responsible for the supervision and funding of the project. All authors reviewed and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Data availability All data supporting the findings of this study are available within the article and its supplementary material.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval Ethical approval was not required for this study as no animals or human participants were involved.

Consent for publication Not applicable. This study does not include any individual person’s data.

Consent to participate Not applicable. This study did not involve human participants.

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