

PRACTICE AND TECHNICAL ARTICLE

Early seedling development and survival of seagrasses *Posidonia australis* and *P. sinuosa* using different seed-based restoration methods

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Seagrass seed-based restoration has been developed in several places worldwide, but disturbance at the vulnerable initial stages of seedling recruitment has proved to be a major bottleneck to successful restoration. A detailed investigation of seagrass seedling survival and growth at the earliest stages of seedling development is an important contribution to developing techniques to improve seedling establishment and survival. Here, we investigated the early seedling development of *Posidonia australis* and *P. sinuosa* as well as quantifying the variation in seedling survival and development under different seed-based restoration methods. Early seedling development was documented in indoor aquaria during the first month after fruit dehiscence. In the second ex situ experiment, we determined the effects of three different restoration methods (surface sowing, seedling planting, and planting inside a hessian bag) on seedling survival and development over the first 2 months of life. In the first experiment, a primary root with the first root hairs developed after 7 days. After 1 month, roots were 20–60 mm in length, firmly establishing the seedling into the sediment. Compared to surface sowing, seedling planting and hessian bag restoration treatments did not significantly affect survival for *P. australis* but increased seedling mortality in *P. sinuosa*, although growth was greater (shoot and root lengths were approximately 50 and 40% longer, respectively). These aquarium-scale experiments suggest that planting methods would enhance seedling establishment at larger scales in some species, promoting restoration of damaged seagrass habitats.

Key words: leaf development, root development, root hairs, seed-based restoration, seedling

Implications for Practice

- Understanding of seagrass early seedling development and survival with seed-based restoration methods is required to develop suitable techniques for restoring seagrass habitats, especially in direct developing seeds of the genera *Thalassia*, *Enhalus*, and *Posidonia*, where seeds show no dormancy and seed banks do not develop.
- *Posidonia australis* and *P. sinuosa* seedlings are very vulnerable to being disturbed by waves and currents during the first few weeks of their life and require environmental manipulation to increase their establishment success.
- Planting seedlings in sediment and inside sand-filled hessian bags can be developed for some Australian *Posidonia* seed-based restoration to improve the success rate of seedling establishment, as these techniques produced larger seedlings although did not enhance survival.

Introduction

Seagrasses are some of the most threatened habitats in the world's oceans despite providing valuable ecosystem services (Short et al. 2011). Concerns about global seagrass decline due to anthropogenic pressures have prompted the emergence of multiple seagrass restoration initiatives using both vegetative (transplants) (van Katwijk et al. 2009) and reproductive (seed) materials (Orth et al. 2020). Seed-based restoration is preferable as it is less

labor-intensive, more cost-effective, and potentially enhances the genetic diversity and resilience in the restored area (Ambo-Rappe 2022). Different seed-based restoration methods have been developed globally, including seed injection (Govers et al. 2022), seed burial in hessian bags (Tan et al. 2023), and burial of seed-balls, where seeds were wrapped in a mud-clay ball and buried in the sediment (Xu et al. 2023). The largest and most successful seed-based restoration program to date is *Zostera marina* (eelgrass) restoration using a direct broadcast seeding technique in Virginia's coastal bays, United States (Orth et al. 2020). Since the early 1990s, 74.5 million seeds have been

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dispersed, resulting in 3612 ha of seagrass meadows (Orth et al. 2020). However, elsewhere, low success rates in seed-based restoration programs resulted from early recruitment bottlenecks (e.g. Statton et al. 2017; Govers et al. 2022).

As seagrass seeds are important in recovery dynamics at the landscape scale, an understanding of how developing seedlings colonize submerged environments will be important to develop successful restoration strategies. Many seagrasses have persistent seeds that form a seed bank, but, some genera, including *Posidonia*, produce direct-developing seeds (recalcitrant seed with precocious germination) (Kendrick et al. 2019). These genera produce positively buoyant fruits, each containing negatively buoyant seeds that germinate before being released from the fruit and directly develop into a seedling (Statton et al. 2013). These seedlings are large (1.5–2.5 cm in length) and settle onto the sediment surface and need to develop roots to anchor into the substratum rapidly to withstand constant water movement (Kendrick et al. 2019). However, our knowledge of early life-stage development in direct developing seeds is restricted to a few seagrass species, such as *Posidonia oceanica* (Guerrero-Meseguer et al. 2018) and *Thalassia hemprichii* (Soong et al. 2013).

Posidonia australis and *P. sinuosa* are endemic species to southern Australia (Short et al. 2011), where they are broadly distributed and form extensive meadows (e.g. Sinclair et al. 2023). They are also the main meadow-forming species in Cockburn Sound, Western Australia (Kendrick et al. 2002), where they suffered extensive loss since 1967 due to excessive nutrient loads associated with industrial pollution (Cambridge and McComb 1984). Seedling establishment from seed-based restoration programs in Cockburn Sound was less than 5% with significant losses occurring within the first 2 months (Statton et al. 2017). Much of the seedling mortality reported in the field is through physical and biological disturbances of seedlings before roots develop and anchor into the sediment. Aquarium experiments would control both physical (currents and waves) and biological disturbances and would allow a test of the effects of burial alone on survival and growth of young seedlings. High mortalities in early seedling development are common in other seagrass species and can result in restoration failure (Govers et al. 2022). Also, developmental studies of *P. oceanica* seedlings demonstrated that rapid shoot and root development in the first 2 months of life strongly influences seedling recruitment success (Guerrero-Meseguer et al. 2018).

This study aimed to investigate aspects of early seedling development and survival of Australian *Posidonia* to improve seedling establishment in seed-based restoration. The specific objectives of this study were to document the early seedling development and establishment of *P. australis* and *P. sinuosa* and to test whether there is any advantage in survival and growth of seedlings when buried.

Methods

Seedling Collection

Mature *Posidonia australis* and *P. sinuosa* fruits were collected November and December 2023 from Woodman Point (32°7'44"S, 115°44'57"E), Southern Flats (32°15'3"S,

115°42'48"E), Carnac Island (32°7'15"S, 115°39'58"E) and South Beach near Fremantle (32°4'22"S, 115°45'2"E), Western Australia. After fruit dehiscence, released seedlings were collected and transported to the laboratory on the same day.

Experimental Design and Setup

In the first experiment, we investigated early seedling development for 28 days. Our timing of sampling was designed to capture initial root development in seedlings of *P. australis* ($n = 174$) and *P. sinuosa* ($n = 124$). These seedlings were placed via broadcast seeding onto the sediment surface in a perspex aquarium (60 cm × 40 cm × 40 cm) filled with a recirculating flow of natural seawater (salinity approximately 33‰, 18–23°C). The light was provided by overhead fluorescent lights (14-hour daylight cycle), delivering 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The sediment consisted of a 15 cm layer of coarse river sand capped with a 1 cm layer of unsorted calcareous sediment sourced from one sampling site (Woodman Point). As the genus *Posidonia* has large (1.5–2 cm) direct-developing seeds, there is no seedbank, so sediment did not need pre-treatment.

In the second experiment, we investigated the effect of different seed-based restoration methods on the survival and growth of *P. australis* and *P. sinuosa* seedlings at weekly intervals over 8 weeks. The experiment followed a block design with tanks nested by sumps. Each tank was split into halves with 50 seedlings each of both species ($\text{Pa} = \textit{Posidonia australis}$, $\text{Ps} = \textit{Posidonia sinuosa}$), which were either the control or the treatments (Fig. S1). The control mimicked the natural process of broadcasted seedlings settling on the sediment surface (Fig. 1A). There were two treatments: planting seedlings in the sediment (bare planted) and in sediment in hessian bags (hessian planted) (Fig. 1B). Fifty seedlings were placed in each of three replicates for control and two burial treatments, resulting in a total of nine tanks (Fig. 1D). The tanks were organized into groups of three, with each group connected to a common sump for water circulation.

Measurement and Harvesting

Documentation of early seedling development in the first experiment was made at 0, 2, 6, 12, 24, 36, 48, and 72 hours after dehiscence from the fruit to describe root emergence and then 5, 7, 14, and 28 days to capture development of the root and root hairs. Ten seedlings were haphazardly selected from the aquarium at each time interval and then photographed and measured for shoot (plumule) and root (radicle) lengths using a caliper to 0.02 mm resolution. Afterwards, the seedlings were fixed using glutaraldehyde (2.5% in seawater with 0.025 M phosphate buffer) and were observed under a dissecting microscope (Nikon SMZ800N) to assess the timing of the development of primary roots and root hairs.

The second experiment examined seedling survival and development under three restoration treatments over an 8-week period. Eight weeks was determined as the early development phase from our field studies of recruiting seedlings (Statton et al. 2017; Kendrick et al. 2023). The number of seedlings that survived was counted every week from week 0 to week

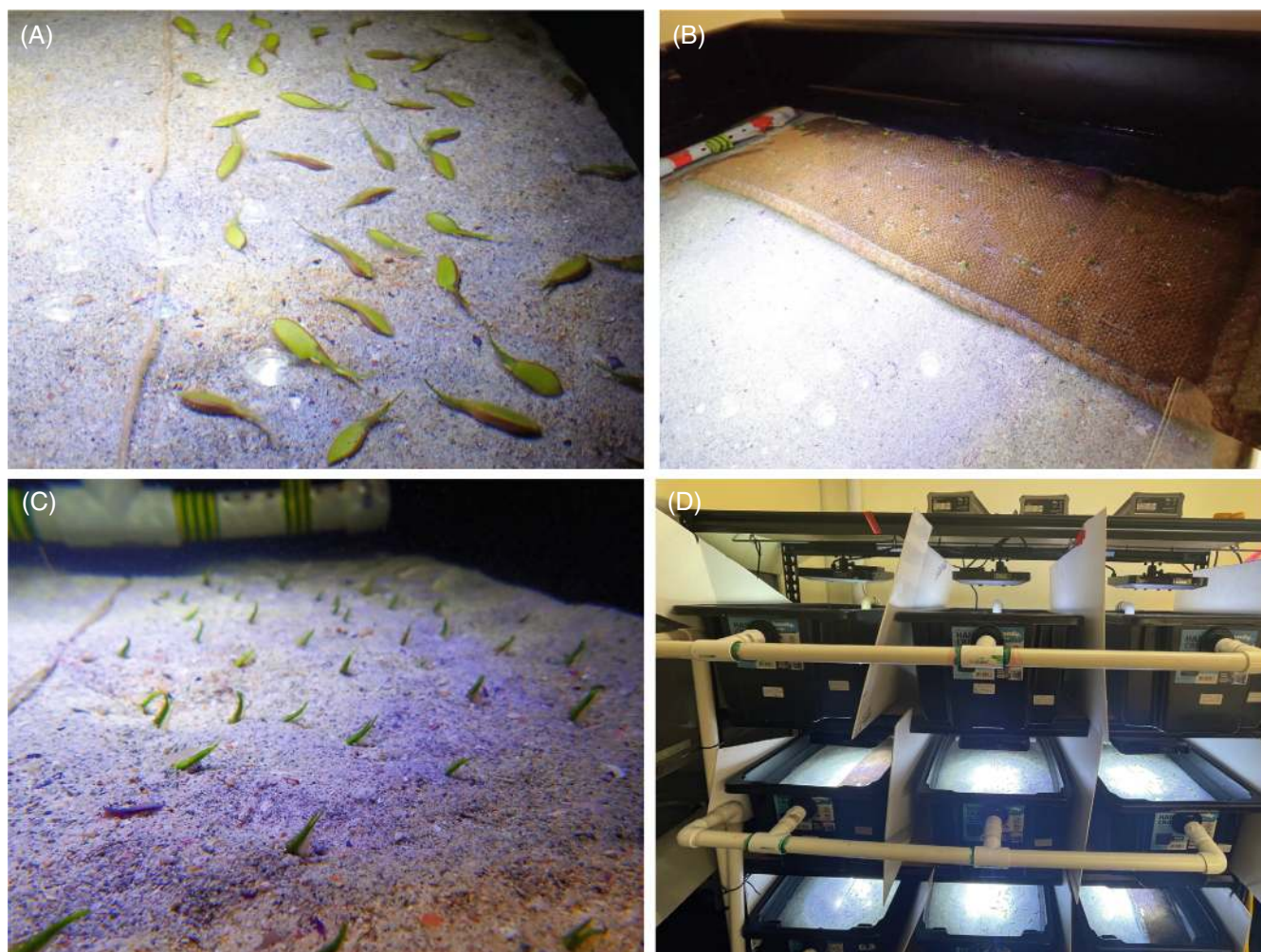


Figure 1. Experiment 2 setup: (A) control: broadcast seeding where seedlings settled horizontally on the surface of sediment; (B) planting in the sand-filled hessian sandbags: seedlings were planted at an angle (with the shoot facing up) in the hessian sandbag (45 cm × 15 cm) filled with beach sand from Woodman Point. Before the seed implantation, the sand-filled bags were soaked in the seawater overnight; (C) seedling planting: seedlings were planted at an angle (1–1.5 cm depth) with shoot facing up; (D) the overall setup for experiment two, each row consisted of three different methods and was connected to one sump to provide water circulation (Figure S1).

8. Seedling mortality was defined as absence of growth between weeks, and seedlings, including the embryo, were dark in color and necrotic. Each week, 10 seedlings of each species from each tank were selected haphazardly, and leaf length and leaf numbers were measured in situ without disturbing the seedlings in the tanks. After 8 weeks, 10 seedlings were harvested and measured for number of leaves produced per seedling, leaf length of the longest leaf per seedling (leaf length in mm), leaf area (leaf length × leaf width) of the largest leaf per seedling (in mm²), longest root per seedling (root length in mm), and number of roots produced.

Data Analysis

Species were tested separately in all statistical analyses in both experiments. Descriptive analysis was used to describe the early seedling development in the first experiment. In the second

experiment, survival analysis for each species was tested separately using the Cox mixed-effects model in R and visualized using Kaplan–Meier curves (Therneau & Grambsch 2000). This model enabled us to accommodate the hierarchical structure of the data, where seedlings were nested in tanks, and tanks were nested in sumps with treatments applied at the tank level, as well as treating the right censored data. The significance level of the statistical tests conducted was $\alpha = 0.05$. The model can be specified as the following:

$$\text{coxme}(\text{Surv}(\text{time}, \text{status}) \sim \text{treatment} + (1|\text{sump}/\text{tank}))$$

Additionally, linear mixed-effect models (LMMs) from the nlme package in R (v3. 1-152; Pinheiro et al. 2021) were used to test the effects of treatment (fixed) on leaf length, leaf area, and root length after 8 weeks. The LMMs included random effects nested within tanks and sumps. Errors of fitted LMMs

were tested for the assumption of normality using Shapiro–Wilk test. Finally, generalized mixed-effect models (GLMMs) with Poisson distribution tested the effect of treatments on the number of leaves and roots per seedling after 8 weeks. The significance level of the statistical tests conducted was $\alpha = 0.05$. To test the statistical significance of the differences between treatments, we computed estimated marginal means (EMMs) using the “emmeans” function in R. The models were:

Linear mixed models (LMMs)

: lme(variables ~ treatment, random = ~ 1 | sump/tank)

Generalized linear mixed

–effect models (GLMMs) : glmer

(variables ~ treatment + [1 | sump/tank], family = Poisson)

Results

Early Seedling Development

Posidonia australis and *P. sinuosa* seeds germinate within the fruit, and all our replicates were seedlings, indicated by the presence of a radicle (first root) and a plumule (first leaf) (Fig. 2). In the first experiment, there were no significant changes in *P. australis* seedling morphology within the first 36 hours after settlement, with mean length of shoot and root of 9.2 ± 1.9 mm SD and 1.9 ± 0.7 mm SD, respectively (Fig. 3A). A root cap formed on the apex of the radicle after 48 hours, becoming more distinctive after 5 days. A few root hairs began to appear on the zone of differentiation of the primary root after 7 days and became denser with sediment grains attached on day 14. At 1 month, another primary root had grown, and *P. australis* seedlings had successfully anchored their roots into the sediment with root lengths of up to 60 mm.

Posidonia sinuosa seedlings developed more slowly than *P. australis*. No significant changes were seen during the first 72 hours after settlement, with shoot and root mean length of 10.9 ± 1.6 mm SD and 1.0 ± 0.7 mm SD, respectively (Fig. 3B). The emergence of root hairs in *P. sinuosa* was less than in *P. australis*. One week after settlement, *P. sinuosa* seedlings developed fewer and shorter root hairs than *P. australis*, but they became denser with sediment grains attached on day

14. After 1 month, the root hair density of *P. sinuosa* was similar to that of 1-month-old *P. australis*. *Posidonia sinuosa* seedlings had also successfully anchored their roots into the sediment with root lengths of up to 47 mm.

Seed-Based Restoration Methods Experiment

Seedling Survival. Mortality of seedlings occurred consistently across burial treatments and control, and average seedling survival of *P. australis* and *P. sinuosa* was $97 \pm 2.65\%$ SD and $72.3 \pm 9.90\%$ SD, respectively (Fig. 4). The output of the Cox mixed-effect model showed no significant differences in seedling survival of *P. australis* between different treatments and control ($p = 0.244$, degrees of freedom [df] = 2). In contrast, the seedling survival of *P. sinuosa* significantly declined in planting treatments compared to control ($p = 0.024$, $df = 2$). Timing of the onset of mortality was different among species: in *P. australis*, mortality occurred after 3 weeks in all treatments, while *P. sinuosa* seedling mortality consistently occurred from the first week in treatments and control (Fig. 4B).

Leaf Development and Morphology. The GLMM (family = Poisson) test results showed that the number of leaves per seedling was not significantly different between treatments and control after 8 weeks for either *P. australis* ($p = 0.550$, $df = 2$) or *P. sinuosa* ($p = 0.412$, $df = 2$) (Fig. S3). Although not statistically significant, the leaf number of both *P. australis* and *P. sinuosa* in the first 3 weeks of the planting treatments increased faster than the control (Fig. S2). Length of the longest leaf per seedling ranged between 46 and 140 mm. GLMMs indicated that seedling leaf length in the planting treatments was significantly greater than that of control groups after 8 weeks for both *P. australis* ($p = 0.003$, $df = 2$) and *P. sinuosa* ($p = 0.010$, $df = 2$). Seedlings in planting treatments consistently produced greater leaf lengths, by about 50%, than controls over the experiment period (Fig. 5, S6, S7). Leaf area (leaf length $\times$ leaf width) of the largest leaf per seedling was also significantly greater in seedlings of planting treatments than those of controls in *P. australis* ($p = 0.003$, $df = 2$) and *P. sinuosa* ($p = 0.015$, $df = 2$) (Fig S4).

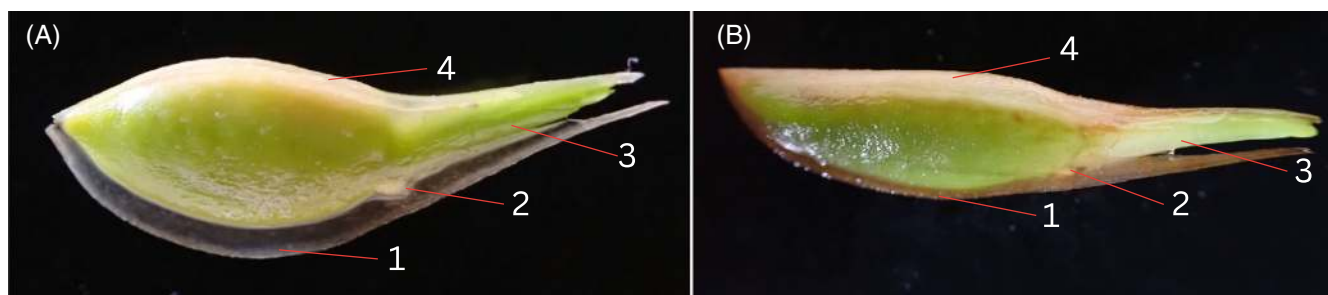


Figure 2. Seedling morphology after fruit dehiscence of *Posidonia australis* (A) and *P. sinuosa* (B): (1) membranous wing (keel), (2) radicle, (3) shoot (profile), and (4) seed.

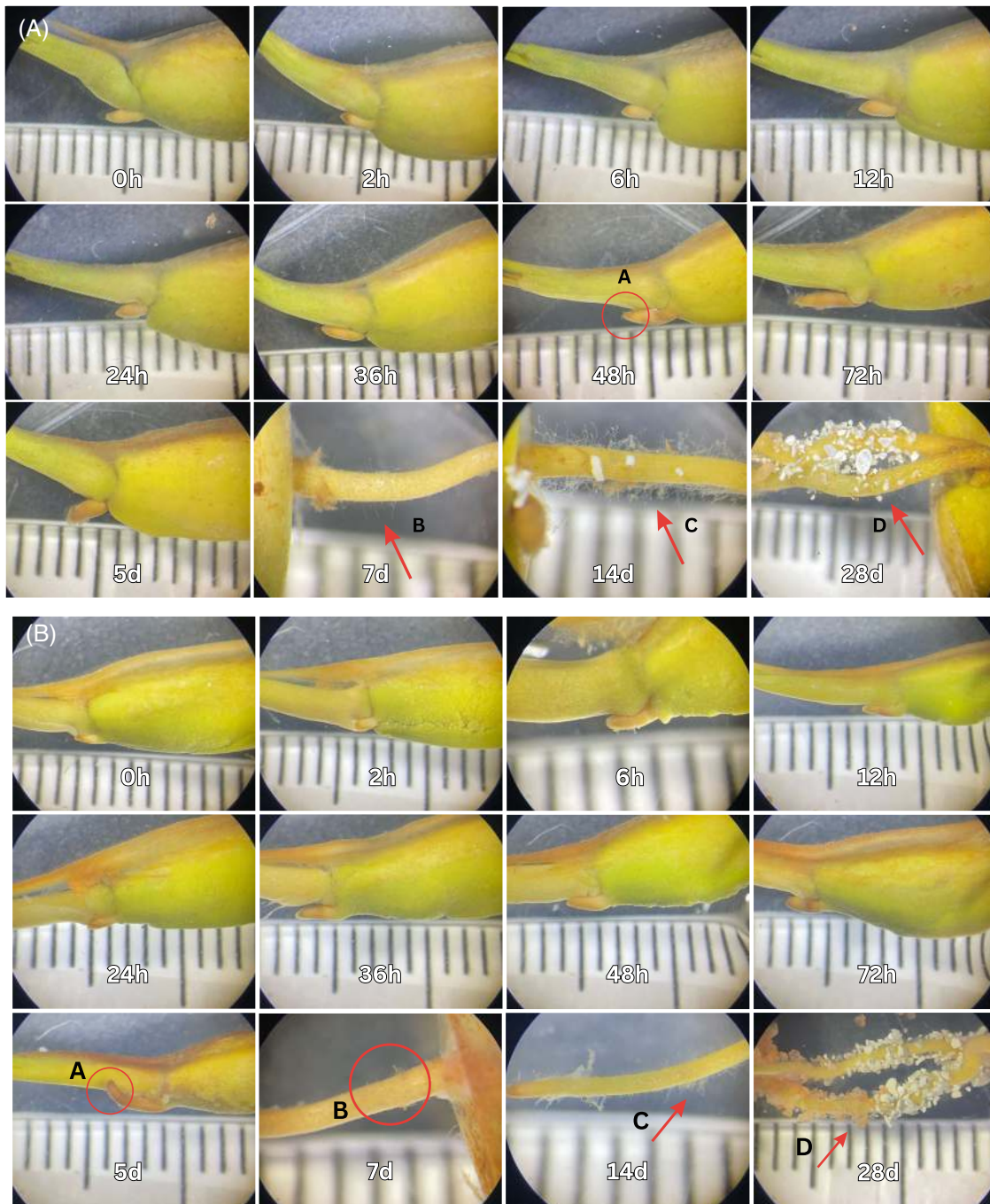


Figure 3. Primary root development observed during experiment 1 for: (A) *Posidonia australis* showing: (a) Cell division to form a root cap began after 36 hours; (b) The adhesive root hairs had grown and were observed on day 7; (c) The surface of the differentiation zone of the primary root was entirely covered with adhesive root hairs, which started to trap some sediment grains; (d) Growth of the second primary root with adhesive root hairs, and for (B) *P. sinuosa* showing (a) Cell division to form a root cap began after 72 hours; (b) The adhesive root hairs began to grow and were observed on day 7; (c) The surface of the zone of maturation of the primary root was covered with adhesive root hairs; (d) Growth of second primary root with adhesive root hairs. Scale bars are in mm, h = hours, d = days.

Root Development and Morphology. Root length of the longest root per seedling after 8 weeks varied between 22 and 172 mm. *Posidonia australis* seedlings of the planting treatment groups showed significantly greater root length than that of controls ($p = 0.002$, $df = 2$), by almost 40% greater (Fig. 6, S6). In

contrast, there was no significant difference in root length of the longest root per seedling of *P. sinuosa* ($p = 0.253$, $df = 2$) (Fig. S7). The number of roots produced after 8 weeks varied between 1 and 4 and was not significantly different for either species (*P. australis*, $p = 0.243$, $df = 2$; *P. sinuosa*,

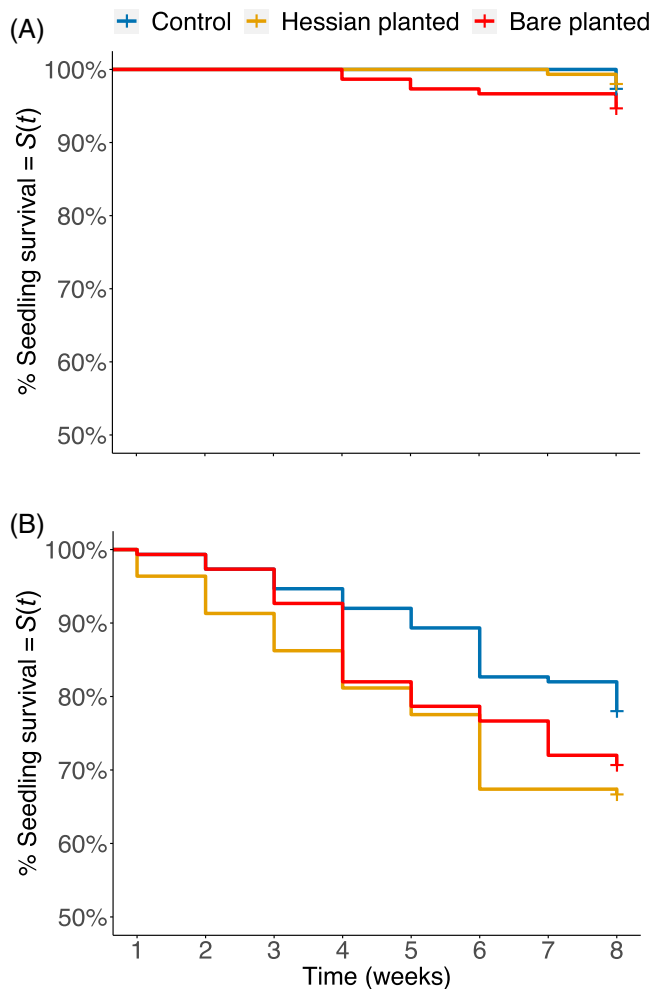


Figure 4. Kaplan-Meier curves of percent seedling survival over 8 weeks of (A) *Posidonia australis* and (B) *P. sinuosa*. Right censoring data are indicated by the horizontal bar at the end of the curves.

$p = 0.347$, $df = 2$). Lateral roots were also observed developing in seedlings from planting treatments but not in the control (Fig. S6).

Discussion

Seagrass seed-based restoration has been developed in several places worldwide (Orth et al. 2020; Sinclair et al. 2021), but disturbance at the vulnerable initial stages of seedling recruitment has proved to be a major bottleneck to successful restoration (Statton et al. 2017). Here, we investigated the early seedling development of *Posidonia australis* and *P. sinuosa*, which is an important contribution to developing techniques to improve seedling establishment and survival in these species. Our findings showed that it takes 7 days for *P. australis* and *P. sinuosa* roots to begin to develop root hairs. During this earliest life stage, the unanchored seedlings settle onto the sediment surface and are vulnerable to disturbance by waves and currents, but after 1 month, seedlings had established a root system, with

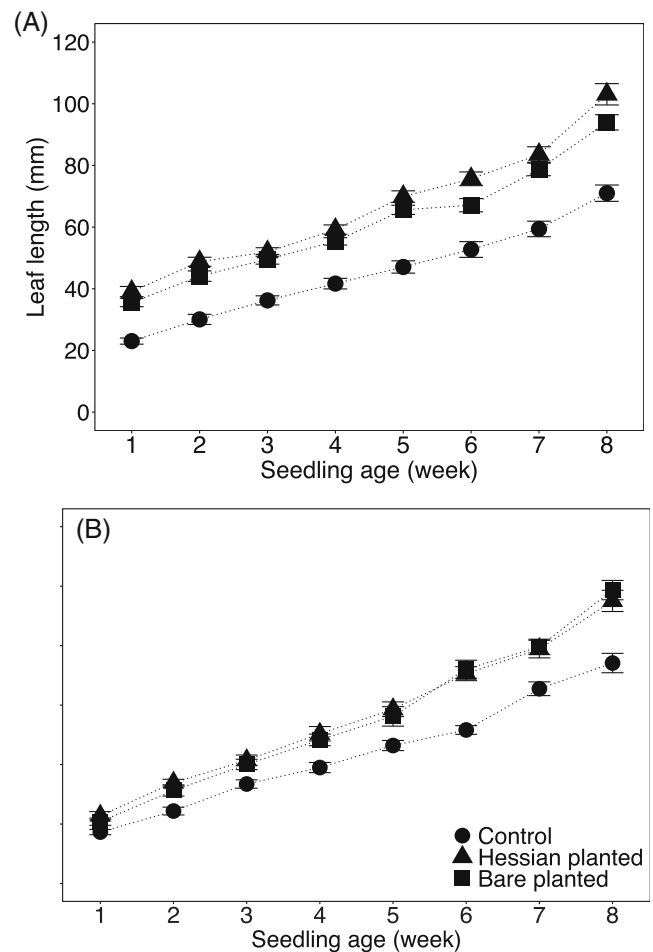


Figure 5. Leaf length of the longest leaf per seedling of *Posidonia australis* (A) and *P. sinuosa* (B) over 8 weeks. Values are means $\pm$ SE, $n = 30$ (seedlings per treatment).

one to several roots penetrating the sand substrate as much as 60 mm. We also compared the effects of burying seedlings that have been previously used in seed-based field trials with varying degrees of success (Statton et al. 2017), examining the survival and development of *P. australis* and *P. sinuosa* seedlings during their first 2 months of life. Burying seedlings in sediment and inside sand-filled hessian bags increased the seedling size by 40%, although survival was significantly reduced in *P. sinuosa*. The reduction in survival may be correlated with declines in oxygen and increased sulfur in laboratory-contained sediments, but this would require further research to validate. Still, larger seedlings with longer roots in buried seedlings are likely to be more resistant to disturbance and dislodgement (Statton et al. 2017).

This investigation is the first to document early seedling development in *P. australis* and *P. sinuosa*. Our findings showed that *P. australis* and *P. sinuosa* seedlings produced root hairs on the differentiation zone of the primary root during root hair development. These hairs emerged within 7 days after germination and completely covered the area behind the growing root tip within 14 days. Sediment grains became entangled in root hairs, but the density of root hairs was sparser on the older

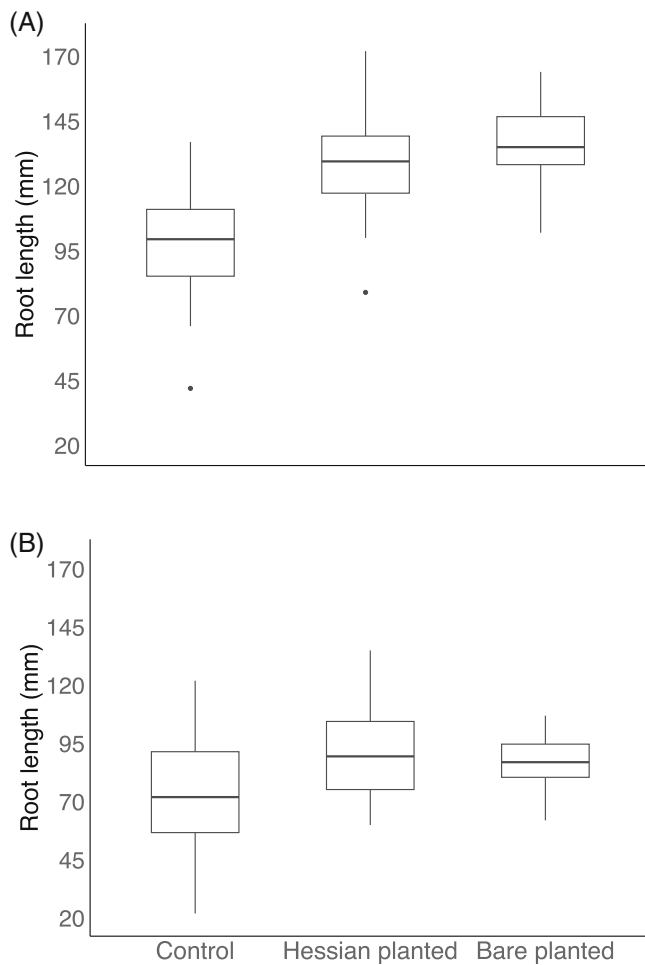


Figure 6. Box plot of length of longest root per seedling of *Posidonia australis* (A) and *P. sinuosa* (B) after 8 weeks. Values are means $\pm$ SE, $n = 30$ (seedlings per treatment).

sections of the root as they developed (Statton et al. 2013). The emergence of root hairs at later stages (e.g. 2 weeks to a month) has been reported in other seagrass species, such as *P. oceanica*, *Thalassia hemprichii*, and *Enhalus acoroides*, but these species also formed a felt-like layer of strongly adhesive hypocotyl hairs on the basal surface of their seeds prior to primary root development (Lacap et al. 2002; Soong et al. 2013; Badalamenti et al. 2015). Hypocotyl hairs facilitated attachment in 2-month-old *P. oceanica* seedlings to coarse gravel and rock (Alagna et al. 2015; Badalamenti et al. 2015; Guerrero-Meseguer et al. 2018). In contrast, *P. australis* and *P. sinuosa* are reliant on a morphological adaptation, a lateral membranous wing for stability before the roots attach. The wing counteracts the tendency of direct developing seeds to roll with water motion on the seabed, enabling roots to grow downwards in response to geotropism (Kendrick et al. 2019).

Seedlings are very vulnerable, and mortality is high within the first weeks to months of seedling development (Statton et al. 2017). The longer the roots take to develop, the higher the risk of seedlings being washed away by waves and currents

(Statton et al. 2017) or being dislodged or consumed by benthic invertebrates (Orth et al. 2006; Johnson et al. 2018). Given the vulnerability of Australian *Posidonia* species during early seed stages of recruitment, manipulating the microenvironment through seedling planting and/or use of sand-stabilizing hessian bags in our controlled laboratory experiment was expected to increase survival and growth. However, there was no evidence that survival was enhanced by the burial treatments, rather that *P. sinuosa* seedling survival was significantly reduced. Sulfur toxicity has been reported from adult *P. sinuosa*, near to our collection location at Woodmans Point (Fraser & Kendrick 2017). *Posidonia sinuosa* is more sensitive to deoxygenation and sulfur toxicity with low current velocities (e.g. Olsen et al. 2018), such as would also be experienced in an aquarium. Burial at a greater depth (4 cm) has previously been shown to increase seedling mortality in the direct developing seeds of *P. australis* (Campbell 2016), whereas burial of seagrass seeds that have delayed germination and form seed banks (e.g. *Cymodocea nodosa*) was reported to increase growth (Marba & Duarte 1994).

Faster growth of leaves and roots resulted in significantly larger seedlings within 2 months for the planting treatments. Roots of *P. australis* (but not *P. sinuosa*) were almost 40% longer after 2 months. Increased root length of buried seeds has also been reported in land plants (Tao et al. 2022). The development of thin lateral roots in earlier studies of *P. australis* transplants has been implicated in increased ability to take up nutrients (Hovey et al. 2012), but stored reserves in the seedling embryo are also adequate to supply leaf and root growth in the first 5 months of development (Hocking et al. 1981; Statton et al. 2013). The development of lateral roots is also important in root system construction to reinforce plant anchoring capacity (Hovey et al. 2012), allowing them to withstand waves and currents (Statton et al. 2017).

The present study provides evidence that the time it takes for shoot and root development in *P. australis* and *P. sinuosa* is likely to make their seedlings vulnerable to water motion during the first few weeks of their life. Planting treatments at a small scale in the aquaria produced larger seedlings but had negative effects on the survival of *P. sinuosa*. This suggests that these methods could be developed to increase seedling establishment in *P. australis* but not *P. sinuosa* at larger scales, promoting restoration of damaged seagrass habitats. Methods that insert seedlings in sediments should be explored for seed-based restoration for direct developing seeds, further given the contrasting effects observed between the two species studied here.

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Supporting Information

The following information may be found in the online version of this article:

Figure S1. The design of the seed-based restoration experiment setup.

Figure S2. *Posidonia australis* and *Posidonia sinuosa* mean number of leaves after 1 week. $n = 3$, $N =$ number of leaves.

Figure S3. *Posidonia australis* (a) and *P. sinuosa* (b) number of leaves per seedling over 8 weeks.

Figure S4. Leaf area of the largest leaf per seedling (leaf length $\times$ leaf width) of *Posidonia australis* and *P. sinuosa* (b) after 8 weeks across three treatments.

Figure S5. Number of roots per seedling of (a) *Posidonia australis* and (b) *P. sinuosa* after 8 weeks across three treatment.

Figure S6. Harvested seedlings of *P. australis* after 8 weeks with three different treatments.

Figure S7. Harvested seedlings of *P. sinuosa* after 8 weeks with three different treatments.

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