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Influence of substrate and burial on the development of Posidonia oceanica: implications for restoration

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Keywords:	seedling, root, leaf, development, seagrass

TITLE: Influence of substrate and burial on the development of *Posidonia oceanica*: implications for restoration.

RUNNING HEAD: Substrate effects on *Posidonia oceanica* seedlings

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AUTHORS CONTRIBUTIONS:

LGM, CSL, AM conception and design of the study; LGM, CSL, KSU, AM acquisition, analysis and interpretation of the data; LGM, CSL drafting of the article; LGM, CSL, KSU, AM revision and approval of the version to be submitted.

ABSTRACT: *Posidonia oceanica* is one of the few seagrasses that can colonize hard and soft substrates. To test whether substrate could affect root development of the seedlings, with a legacy effect upon transplantation to sand, we germinated seeds on hard (glass slide) vs. soft (sand) substrates in microcosms. We found that sand favored root system development, with a compensatory slowing of leaf development, while glass had the opposite effect. After four months, we transplanted all seedlings to sand and tested for a legacy effect of initial substrate type. Leaves of seedlings germinated on sand and glass slides reached approximately the same length, but roots from seedlings germinated on glass did not develop fully. Seed-burial (0 – 1.5 cm) did not affect seedling development. These results suggest that the culture of *P. oceanica* on sand prior to transplantation could enhance seedlings survival in restoration programs.

KEY WORDS: Seedling, root, leaf, development, seagrass.

IMPLICATIONS:

- The culture of *P. oceanica* seedlings on sand promotes the development of the root system.
- The initial substrate has a legacy effect on the subsequent development of seedling transplants.
- Shallow seed-burial (0.5 -1.5 cm) does not affect the development of five-month-old seedlings.

MAIN TEXT:

INTRODUCTION

Posidonia oceanica (L.) Delile is one of the most important habitat-forming species in the Mediterranean Sea (Duarte & Chiscano 1999; Pergent et al. 1997). It has been selected as an indicator species to assess the ecological status of Mediterranean coastal water bodies (WFD, 2000/60/EC; Lopez y Royo et al. 2011) because it supports high biodiversity and plays a key role in several ecosystem functions (e.g. Molinier & Picard 1952, Koch et al. 2009, Duarte et al. 2010, Valle 2011, Sanz-Lázaro et al. 2012).

P. oceanica meadows are currently declining (Boudouresque et al. 2009; Marbà et al. 2014) due to pollution (Cancemi et al. 2003; Balestri et al. 2004) and a range of anthropogenic activities that alter sedimentation rates and consolidation of seabed substrates (Pasqualini et al. 2000; Ruiz & Romero 2003; Badalamenti et al. 2006, 2011; González-Correa et al. 2005, 2008). To mitigate the decline of *P. oceanica* meadows, environmental restoration projects have been undertaken. Recent projects have used laboratory-cultivated seedlings, which has the advantage of promoting genetic variability (Balestri et al. 1998; Terrados et al. 2013). However, this type of restoration has anchorage problems. Even though seedlings are capable of remaining anchored in different substrates (Badalamenti et al. 2015; Balestri et al. 2015), their roots are not long enough to adhere firmly (Balestri & Bertini 2003), so seedlings can be uprooted by waves and currents (Meinesz et al. 1993).

Some authors argue that *P. oceanica* seedlings only persist on vegetated rocky substrates, while those in sand and gravel are unable to grow (Alagna et al. 2013). Nevertheless, other studies confirm the establishment of seedlings in sandy bottoms (Balestri and Lardicci 2008, Balestri et al. 2015). In general, the seeds of seagrasses germinate within the sediment, which benefits their growth (Marba & Duarte 1994; Moore et al. 1993; Terrados 1997). The *P. oceanica* seeds have photosynthetic activity (Celdrán & Marín, 2011), which contributes to early development after germination (Celdrán & Marín 2013). It is not known how seed-burial could affect the development of seedlings. Increasing our knowledge of *P. oceanica* establishment on different substrates and effects of seed-burial is key to improving restoration of this plant and, consequently, supporting management strategies for its conservation.

The aim of this study was to test the effects of substrate hardness and seed-burial on the development of *P. oceanica*. Using microcosm experiments, we tested three interrelated hypotheses. First, substrate hardness could affect the root system development during the first months of germination. Second, the substrate where the seedlings germinate could influence their subsequent development upon transplantation. Third, shallow burial of the seed could decrease seedling development, possibly by limiting photosynthesis.

MATERIALS AND METHODS

Culture of *P. oceanica* seedlings

P. oceanica buoyant fruits were collected in May 2013 from beaches in Ibiza island (Spain, western Mediterranean Sea). Seeds were manually extracted and germinated in aquaria with artificial seawater prepared with sterilized bi-distilled water and marine salt (Ocean Fish, PRODAC International, Cittadella, Italy). Seawater had a salinity of 36 psu and a temperature of 21 ± 1 ºC. The average photosynthetic photon flux density on the surface of seedling leaves was 100 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent lights, with a 14:10 h light:dark photoperiod.

Effects of substrate hardness on seedling development

To test the effects of substrate hardness on the early stages of growth of *P. oceanica* seedlings, seeds of similar size (1.65 ± 0.2 cm length) were placed into replicate 10 L aquaria in plastic pots (9 x 9 x 10 cm) containing glass slides or sand (n=30).

The effects of sand were tested using pots filled to 10 cm depth with sand collected from an unpolluted area close to the marine reserve of Cabo de Palos-Islas Hormigas (Murcia, Spain). The sediment was composed of 32% coarse-sand, 67% of fine-sand according to the Wentworth (1992) scale of particle size, 0.75% organic matter and a C:N ratio of 13.9 : 3.67. To simulate hard substrate, glass microscope slides were placed over the sand in the pots. Glass was chosen as the hard substrate because it is an inert material of known chemical composition, with a constant roughness and structure.

The experiment was performed in a culture chamber room under controlled temperature, salinity and photoperiod conditions. Water level and salinity within aquaria were checked every three days, and aeration was adjusted to supply dissolved oxygen without disturbing the sediment. Aquaria were refilled to compensate for evaporation and maintain salinity of 36 psu. Aquaria were maintained at 21±1 °C, with a 14:10 h light: dark photoperiod and a light intensity of 100 μmol m^2 s^{-1}. The redox potential of the sand was measured with an Orion ORP 91-80 electrode prior to calibration with a redox buffer solution (220 mV at 25 ºC). Measurement of the sediment redox was performed by randomly taking four cores from the sediment-collection area and inserting the electrode to a depth of ca. 4 cm. The sand used as a unconsolidated substrate had a positive redox potential in all pots (+84.5 ± 6.9 mV). Aquaria were aerated to avoid changes in redox potential during the experiment.

The lengths of leaves and roots (principals and laterals) were measured after four months.

Legacy of initial substrate on seedling development and responses to seed-burial

To evaluate the influence of shallow seed-burial and the legacy of substrate hardness, we simulated a restoration event: 60 four-month-old seedlings from the previous experiment were transplanted to sand. Seedlings were carefully removed from the initial substrates by hand, to avoid damaging roots.

We used a factorial design with two fixed factors, *initial substrate* (glass vs. sand) and *seed-burial level* (non-buried, half-buried and full-buried). For the “non-buried” treatment, the seedling were anchored on the sand only by their roots, so each seed was totally uncovered and exposed to light. For the “half-buried” treatment, the seed was partially covered (0.5 cm). In the “full-buried” treatment, the seed was covered by sand (1.5 cm) with only the leaves unburied. The sand over the seed reduced light intensity to 1.45 ± 0.01 µmol m^2 s^{-1} (Fig. 1).

Seedlings were planted in plastic pots (9 x 9 x 10 cm) and placed in individual 10 L aquaria (n= 10), which were maintained in a culture chamber room with the same controlled conditions and sand characteristics as in the previous experiment. The lengths of seed, leaves and roots (principal and lateral) of each seedling were measured at the start of the experiment and again after one month (October 2013) to calculate the net growth of leaves and roots (principal and laterals) per seedling.

Data analysis

Data normality and homogeneity of variance were tested using *P-P* plots and Levene's test, respectively. If data did not meet parametric assumptions, they were transformed [ln (x + 1)], and re-tested. If data still did not meet homogeneity of variances, a significance threshold of $p<0.01$ was assigned, which is a conservative option considering the high number of total replicates (Underwood 1997). Otherwise, a significance threshold of $p<0.05$ was used.

To test the effects of substrate hardness on seedling development, a Student’s *t*-test was carried out to evaluate the possible effects of the fixed factor *initial substrate* on the length of roots (principal and lateral) and leaves. A two-way factorial analysis of variance (ANOVA) was used to evaluate the effects of *initial substrate* and *seed-burial level*, and their possible interaction, on growth of leaves and principal and lateral roots. Additionally, linear regression analysis tested whether seed size influenced the growth of leaves and roots. Data are reported as mean ± standard error (SE). All statistical analyses were carried out using R (v. 3.1.1).

RESULTS

Effects of substrate on seedling development

There were significant differences between substrates ($p < 0.001$) in the total length of principal roots and in the number and length of lateral roots. The roots of seedlings germinated on sand (mean = 17.0 ± 5.4 cm) were up to five times longer than those germinated on glass (mean = 3.7 ± 1.7 cm; Fig. 1, 2).

Total leaf growth had the opposite trend: leaves of seedlings cultured on the glass averaged 46.8 ± 7.9 cm per seedling, which was significantly greater than on sand (35.6 ± 10.6 cm per seedling; Fig. 2).

Seedlings germinated on glass did not have lateral roots, while on the sand lateral roots were found on 70% of seedlings. Lateral root length varied, ranging from 0.1 to 3.3 cm (Fig. 3).

Legacy of initial substrate on seedling development and responses to seed-burial

The growth rates of leaves and roots were not influenced by seed size (p-value of the regression = 0.96 and 0.26, respectively). Shadow seed-burial did not affect leaf or root growth rate ($p = 0.39$ and 0.07, respectively; Table S1). However, seedling development appeared to be influenced by their initial substrate. After the restoration simulation, the growth rates of leaves and lateral roots were significantly higher ($p < 0.01$) in seedlings that had originally developed in sand. In contrast, the growth rate of principal roots was not affected by the initial substrate ($p = 0.71$; Fig. 4).

Leaves of seedlings germinated on sand grew 2.6 ± 0.9 cm $\cdot$ leaf^{-1} $\cdot$ month^{-1}, while those on glass grew 1.9 ± 0.6 cm $\cdot$ leaf^{-1} $\cdot$ month^{-1}. At the end of the experiment, the leaves of the seedlings that initially developed on glass were as long as those germinated on sand (9.1 ± 1.4 and 9.3 ± 1.9 cm, respectively). The growth rate of lateral roots on seedlings germinated on sand was four times greater than those germinated on glass (Fig. 4). The production of new lateral roots per seedling was significantly greater on sand than on glass ($p < 0.001$).

At the end of both experiments, the sediments in all pots had a positive redox potential.

DISCUSSION

P. oceanica showed a high morphological plasticity to two substrates after germination. Sand seemed to promote the growth of principal and lateral roots during the initial four months of plant development. Moreover, the use of sand in germinating *P. oceanica* had legacy effects on subsequent leaf and root development after transplantation to sand. Extensive root growth on sand appeared to be at the expense of leaf development. A similar leaf-root trade-off was observed in *P. oceanica* growing on rubble mounds (Di Carlo et al. 2007). However, when seedling were transplanted to sand, all reached same length.

These results agree with field studies where sand promoted the formation of lateral roots, more than on rock (Balestri et al. 2015). The nutrient content and the unconsolidated structure of the sand could enhance root growth of *P. oceanica*, while the hard glass limited elongation and nutrient acquisition. *P. oceanica* seedlings have adhesive root hairs that facilitate anchorage on rocky substrates (Badalamenti et al. 2015). Likewise, roots on glass were fully adhered to the surface in our experiment. *Phyllospadix scouleri* Hook. also has root hairs that help anchor plants to substrates in intertidal zones (Short & Coles 2001; Kuo& Den Hartog 2006).

P. oceanica seeds photosynthesize even after germination (Celdrán & Marín 2011), so it is possible that shallow seed-burial could negatively affect development. Nevertheless, growth of four-month-old seedlings was not influenced by seed-burial. It is likely that the leaves of young *P. oceanica* soon compensate for any reduced photosynthesis by seeds.

The general procedure for restoration of *P. oceanica* is to attach transplants to the substrate using non-biodegradable materials (Augier et al. 1996; Balestri et al. 1998, 2011; Meinesz et al. 1992; Meinesz et al. 1993; Molenaar et al. 1993; Molenaar & Meinesz 1992). A well-developed root system is expected to maximize anchoring capacity and nutrient acquisition (Balestri & Lardicci 2005; Schutten et al. 2005; Statton et al. 2014). Infantes et al. (2011) evaluated the substrate-anchoring capacity of *P. oceanica* seedlings for the hydrodynamic conditions of the Mediterranean and estimated the minimal root length needed by seedlings to prevent dislodgement from sand (0.35 times the square root of the leaf area). Using this equation, 100% of our seedlings on sand and 13% of those on glass would remain attached to a sandy seabed.

Germination of *P. oceanica* seedlings on sand enhanced the growth of principal root and had a legacy of enhanced leaf and lateral root growth after transplantation. However, shallow burial of the seed did not slow the development of five-month-old *P. oceanica* seedlings. Thus, we suggest culturing seedlings on sand before transplantation, to facilitate anchorage and avoid using non-biodegradable materials in seagrass restoration. Transplantation to sand should be limited to periods of calm weather and in bays that are protected from strong hydrodynamic events.

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ARTWORK AND TABLES WITH CAPTATION:

Figure 1. *P. oceanica* seedlings germinated in sand (A) and glass (B). Numbers indicate the shadow seed-burial level used in the restoration simulation: 1: non-buried; 2: half-buried; 3: full-buried. L: leaves; S: seed; Rh: rhizome, R1: principal roots; R2: lateral roots.

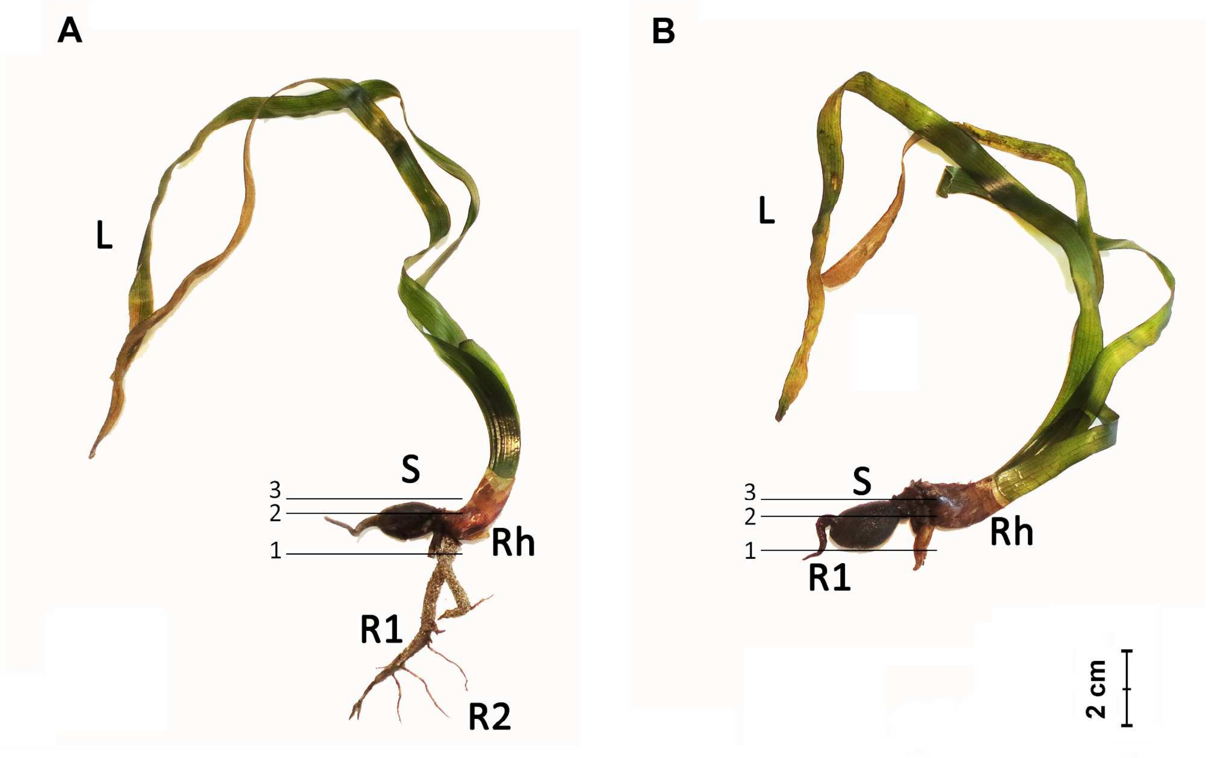


Figure 2. Growth of principal roots and leaves per seedling germinated in sand and glass (mean + SE; n=30). Letters above the bars indicate significant differences between substrate types (Student's *t* - test, $p < 0.05$).

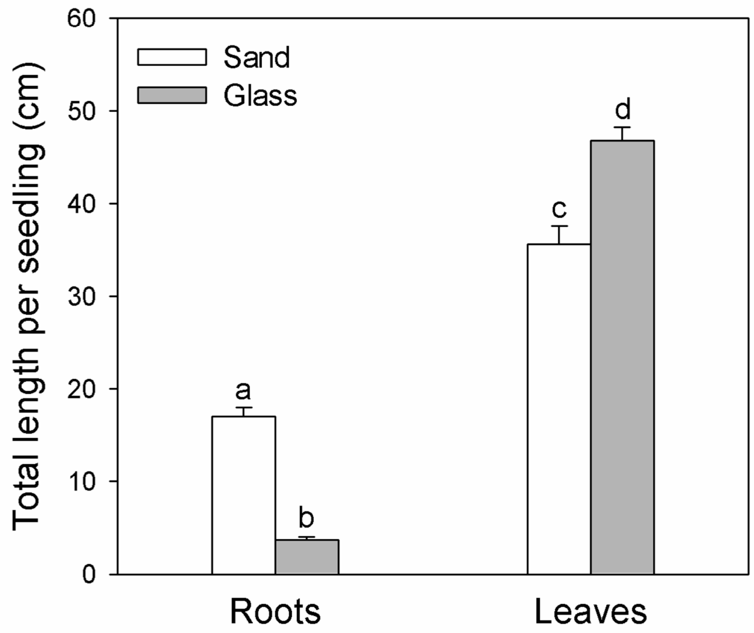


Figure 3. Number of new lateral roots and total length of lateral roots per seedling germinated in sand and glass (mean + SE; n=30). Letters above the bars indicate significant differences between treatments (Student's *t*-test, $p < 0.05$).

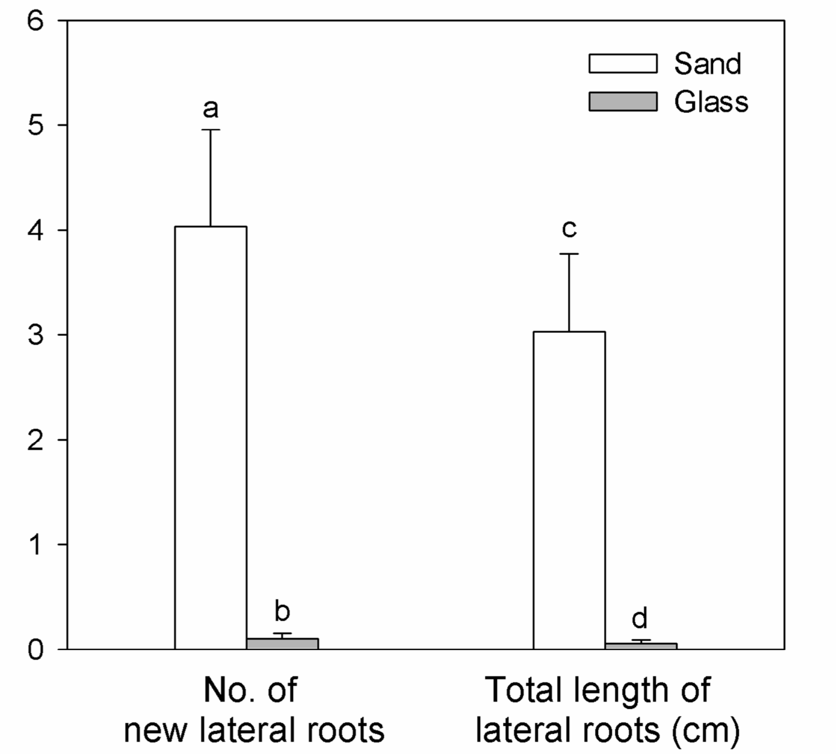
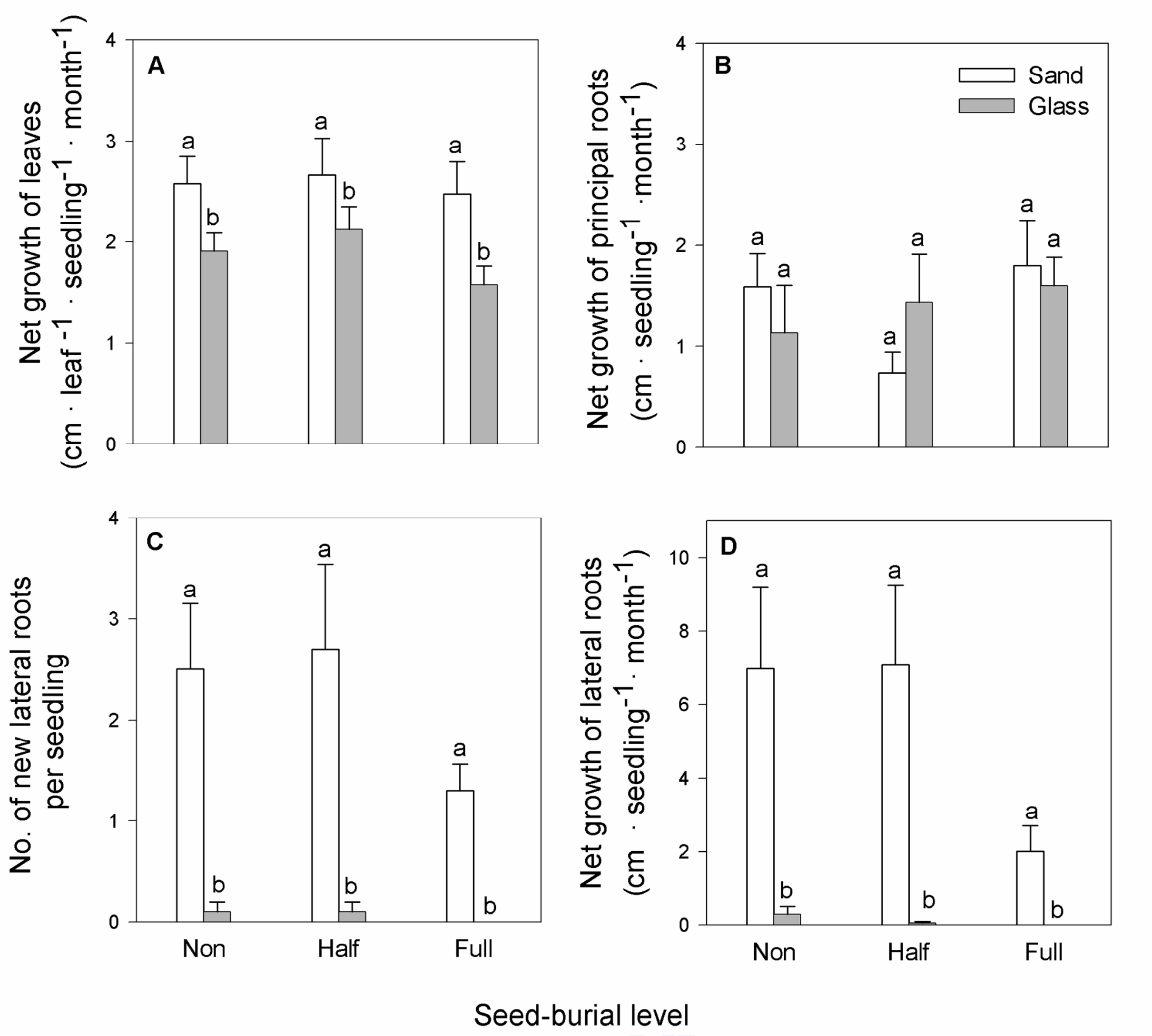
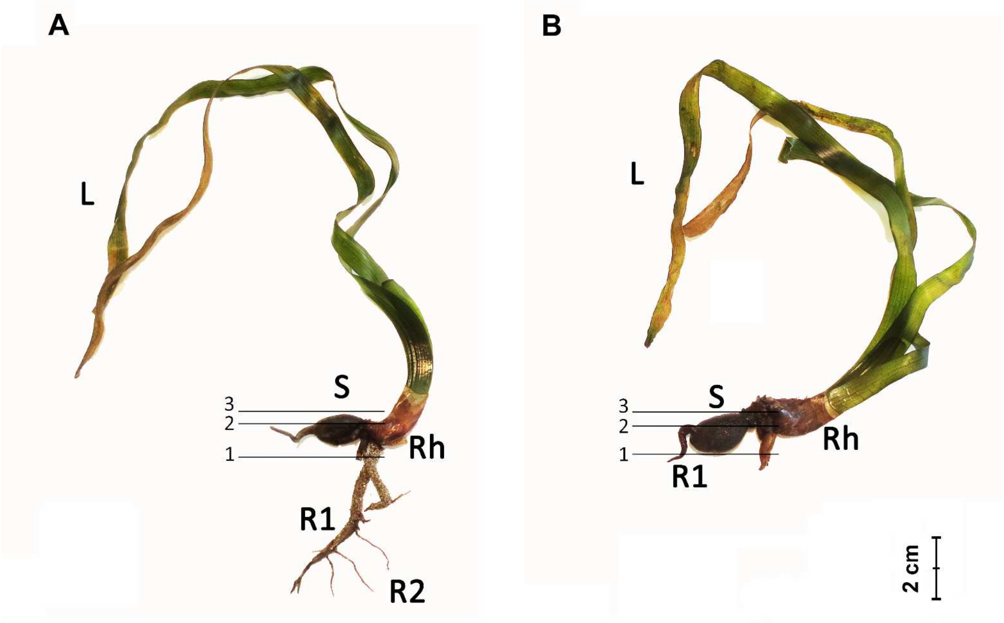


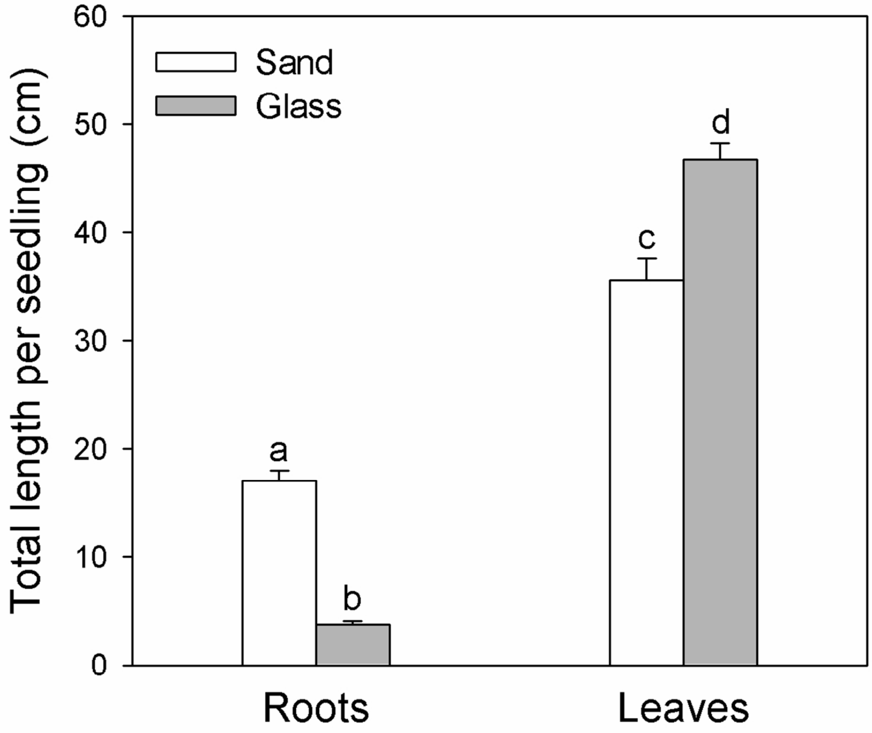
Figure 4. Growth of leaves (A), growth of principal roots (B), number of new lateral roots (C) and growth of lateral roots per seedling (D) after of the restoration simulation for the treatments *Seed-burial level* (non-buried, half-buried and full-buried) and *Initial substrate* (sand in empty and glass in grey; mean + SE; n=10). Letters above the bars indicate significant differences (two-way ANOVA, $p < 0.05$).


A
Net growth of leaves
(cm · leaf -1 · seedling-1 · month-1)
0
1
2
3
4
a
b
a
b
a
b
B
Sand
Glass
Net growth of principal roots
(cm · seedling-1 ·month-1)
0
1
2
3
4
a
a
a
a
a
a
C
No. of new lateral roots
per seedling
0
1
2
3
4
a
b
a
b
a
b
D
Net growth of lateral roots
(cm · seedling-1 · month-1)
0
2
4
6
8
10
a
b
a
b
a
b
Non
Half
Full
Non
Half
Full
Seed-burial level



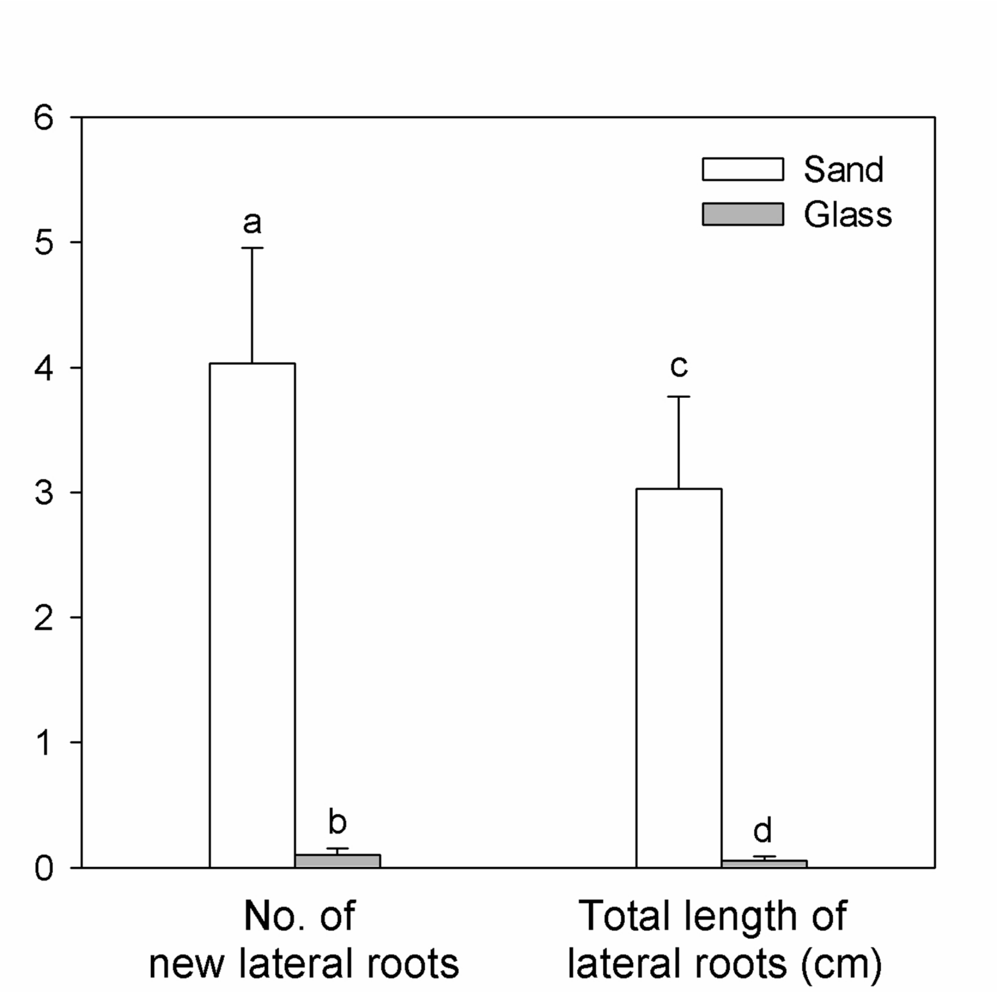
P. oceanica seedlings germinated in sand (A) and glass (B). Numbers indicate the shadow seed-burial level used in the restoration simulation: 1: non-buried; 2: half-buried; 3: full-buried. L: leaves; S: seed; Rh: rhizome, R1: principal roots; R2: lateral roots.

289x194mm (300 x 300 DPI)



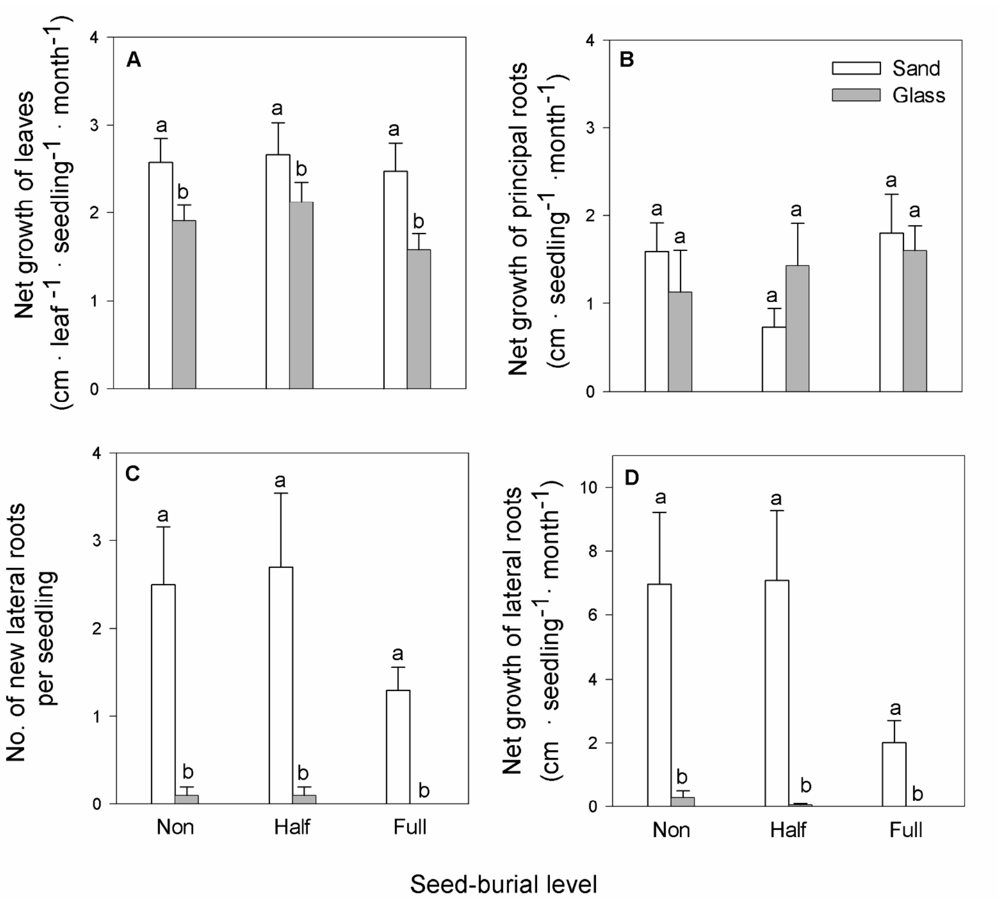
Growth of principal roots and leaves per seedling germinated in sand and glass (mean + SE; n=30). Letters above the bars indicate significant differences between substrate types (Student's t - test, $p< 0.05$).

120x104mm (300 x 300 DPI)



Number of new lateral roots and total length of lateral roots per seedling germinated in sand and glass (mean + SE; n=30). Letters above the bars indicate significant differences between treatments (Student's t-test, $p < 0.05$).

118x116mm (300 x 300 DPI)



Growth of leaves (A), growth of principal roots (B), number of new lateral roots (C) and growth of lateral roots per seedling (D) after of the restoration simulation for the treatments Seed-burial level (non-buried, half-buried and full-buried) and Initial substrate (sand in empty and glass in grey; mean + SE; n=10). Letters above the bars indicate significant differences (two-way ANOVA, p< 0.05).

198x178mm (300 x 300 DPI)

Table S1. Summary of the Levene test and two-way analysis of variance (ANOVA) on the fixed factors “*Initial substrate*” (glass vs. sand) and “*Seed-burial level*” (non-buried, half-buried and full-buried). Significant results are in bold.

Source	**d.f.**	**MS**	***F***	**P**
Growth of leaves				
Initial Substrate (S)	1	7.37	10.35	**<0.01**
Seed-Burial level (B)	2	0.68	0.96	0.39
S x B	2	0.16	0.22	0.80
Residual	54	0.71		
Levene's test:	*p* = 0.36			
Transformation:	None			
Growth of principal roots				
Initial Substrate (S)	1	0.03	0.14	0.71
Seed-Burial level (B)	2	0.65	2.85	0.07
S x B	2	0.41	1.81	0.17
Residual	54	0.23		
Levene's test:	*p* = 0.72			
Transformation:	Ln (x + 1)			
Growth of lateral roots				
Initial Substrate (S)	1	26.22	50.97	**< 0.001**
Seed-Burial level (B)	2	1.29	2.51	0.09
S x B	2	0.95	1.85	0.17

Residual	54	0.51		
Levene's test:	$p = 0.00$			
Transformation:	Ln (x + 1)			
New lateral roots per seedling				
Initial Substrate (S)	1	12.14	60.29	**< 0.001**
Seed-Burial level (B)	2	0.33	1.64	0.20
S x B	2	0.08	0.38	0.69
Residual	54	0.20		
Levene's test:	$p = 0.00$			
Transformation:	Ln (x + 1)			