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

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Complete List of Authors:	Guerrero-Meseguer, Laura; Universidad de Murcia, Departamento de Ecología e Hidrología Sanz-Lázaro, Carlos; Universitat d'Alacant, Departamento de Ciencias del Mar y Biología Aplicada Suk-ueng, Krittawit; Universidad de Murcia, Departamento de Ecología e Hidrología Marín, Arnaldo; Universidad de Murcia, Departamento de Ecología e Hidrología
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Review

1 1 **TITLE:** Influence of substrate and burial on the development of *Posidonia oceanica*: implications
2 2 for restoration.

3 3 **RUNNING HEAD:** Substrate effects on *Posidonia oceanica* seedlings

4 4 **AUTHORS:** Laura Guerrero-Meseguer*¹, Carlos Sanz-Lázaro², Krittawit Suk-ueng¹, Arnaldo
5 5 Marín¹.

6 6 **AFFILIATION AND ADDRESS OF AUTHORS:**

7 7 ¹ Departamento de Ecología e Hidrología. Facultad de Biología. Universidad de Murcia. Campus de
8 8 Espinardo. 30100 Murcia, Spain (corresponding address)

9 9 ² Departamento de Ciencias del Mar y Biología Aplicada, Pabellón 13, Universidad de Alicante,
10 10 P.O. Box 99, E-03080 Alicante, Spain

11 11 **CORRESPONDING AUTHOR:** Laura Guerrero-Meseguer, email address:

12 12 laura.guerrero@um.es, telephone: 00 34 868884977, fax: 00 34 868883963

13 13 **AUTHORS CONTRIBUTIONS:**

14 14 LGM, CSL, AM conception and design of the study; LGM, CSL, KSU, AM acquisition, analysis
15 15 and interpretation of the data; LGM, CSL drafting of the article; LGM, CSL, KSU, AM revision
16 16 and approval of the version to be submitted.

17 17 **ABSTRACT:** *Posidonia oceanica* is one of the few seagrasses that can colonize hard and soft
18 18 substrates. To test whether substrate could affect root development of the seedlings, with a legacy
19 19 effect upon transplantation to sand, we germinated seeds on hard (glass slide) vs. soft (sand)
20 20 substrates in microcosms. We found that sand favored root system development, with a
21 21 compensatory slowing of leaf development, while glass had the opposite effect. After four months,
22 22 we transplanted all seedlings to sand and tested for a legacy effect of initial substrate type. Leaves
23 23 of seedlings germinated on sand and glass slides reached approximately the same length, but roots
24 24 from seedlings germinated on glass did not develop fully. Seed-burial (0 – 1.5 cm) did not affect
25 25 seedling development. These results suggest that the culture of *P. oceanica* on sand prior to
26 26 transplantation could enhance seedlings survival in restoration programs.

27 27 **KEY WORDS:** Seedling, root, leaf, development, seagrass.

28 **IMPLICATIONS:**

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

34 **MAIN TEXT:**35 **INTRODUCTION**

36 *Posidonia oceanica* (L.) Delile is one of the most important habitat-forming species in the
37 Mediterranean Sea (Duarte & Chiscano 1999; Pergent et al. 1997). It has been selected as an
38 indicator species to assess the ecological status of Mediterranean coastal water bodies (WFD,
39 2000/60/EC; Lopez y Royo et al. 2011) because it supports high biodiversity and plays a key role in
40 several ecosystem functions (e.g. Molinier & Picard 1952, Koch et al. 2009, Duarte et al. 2010,
41 Valle 2011, Sanz-Lázaro et al. 2012).

42 *P. oceanica* meadows are currently declining (Boudouresque et al. 2009; Marbà et al. 2014)
43 due to pollution (Cancemi et al. 2003; Balestri et al. 2004) and a range of anthropogenic activities
44 that alter sedimentation rates and consolidation of seabed substrates (Pasqualini et al. 2000; Ruiz &
45 Romero 2003; Badalamenti et al. 2006, 2011; González-Correa et al. 2005, 2008). To mitigate the
46 decline of *P. oceanica* meadows, environmental restoration projects have been undertaken. Recent
47 projects have used laboratory-cultivated seedlings, which has the advantage of promoting genetic
48 variability (Balestri et al. 1998; Terrados et al. 2013). However, this type of restoration has
49 anchorage problems. Even though seedlings are capable of remaining anchored in different
50 substrates (Badalamenti et al. 2015; Balestri et al. 2015), their roots are not long enough to adhere
51 firmly (Balestri & Bertini 2003), so seedlings can be uprooted by waves and currents (Meinesz et al.
52 1993).

1 53 Some authors argue that *P. oceanica* seedlings only persist on vegetated rocky substrates,
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3 54 while those in sand and gravel are unable to grow (Alagna et al. 2013). Nevertheless, other studies
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5 55 confirm the establishment of seedlings in sandy bottoms (Balestri and Lardicci 2008, Balestri et al.
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7 56 2015). In general, the seeds of seagrasses germinate within the sediment, which benefits their
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9 57 growth (Marba & Duarte 1994; Moore et al. 1993; Terrados 1997). The *P. oceanica* seeds have
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11 58 photosynthetic activity (Celdrán & Marín, 2011), which contributes to early development after
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13 59 germination (Celdrán & Marín 2013). It is not known how seed-burial could affect the development
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15 60 of seedlings. Increasing our knowledge of *P. oceanica* establishment on different substrates and
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17 61 effects of seed-burial is key to improving restoration of this plant and, consequently, supporting
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19 62 management strategies for its conservation.
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22 63 The aim of this study was to test the effects of substrate hardness and seed-burial on the
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24 64 development of *P. oceanica*. Using microcosm experiments, we tested three interrelated
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26 65 hypotheses. First, substrate hardness could affect the root system development during the first
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28 66 months of germination. Second, the substrate where the seedlings germinate could influence their
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30 67 subsequent development upon transplantation. Third, shallow burial of the seed could decrease
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32 68 seedling development, possibly by limiting photosynthesis.
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35 69 **MATERIALS AND METHODS**

36 70 **Culture of *P. oceanica* seedlings**

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39 71 *P. oceanica* buoyant fruits were collected in May 2013 from beaches in Ibiza island (Spain,
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41 72 western Mediterranean Sea). Seeds were manually extracted and germinated in aquaria with
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43 73 artificial seawater prepared with sterilized bi-distilled water and marine salt (Ocean Fish, PRODAC
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45 74 International, Cittadella, Italy). Seawater had a salinity of 36 psu and a temperature of 21 ± 1 °C.
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47 75 The average photosynthetic photon flux density on the surface of seedling leaves was $100 \mu\text{mol m}^{-2}$
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49 76 s^{-1} irradiance provided by cool white fluorescent lights, with a 14:10 h light:dark photoperiod.
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52 77 **Effects of substrate hardness on seedling development**

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1 78 To test the effects of substrate hardness on the early stages of growth of *P. oceanica*
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3 79 seedlings, seeds of similar size (1.65 ± 0.2 cm length) were placed into replicate 10 L aquaria in
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5 80 plastic pots (9 x 9 x 10 cm) containing glass slides or sand (n=30).
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7 81 The effects of sand were tested using pots filled to 10 cm depth with sand collected from an
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9 82 unpolluted area close to the marine reserve of Cabo de Palos-Islas Hormigas (Murcia, Spain). The
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11 83 sediment was composed of 32% coarse-sand, 67% of fine-sand according to the Wentworth (1992)
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13 84 scale of particle size, 0.75% organic matter and a C:N ratio of 13.9 : 3.67. To simulate hard
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15 85 substrate, glass microscope slides were placed over the sand in the pots. Glass was chosen as the
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17 86 hard substrate because it is an inert material of known chemical composition, with a constant
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19 87 roughness and structure.
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22 88 The experiment was performed in a culture chamber room under controlled temperature,
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24 89 salinity and photoperiod conditions. Water level and salinity within aquaria were checked every
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26 90 three days, and aeration was adjusted to supply dissolved oxygen without disturbing the sediment.
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28 91 Aquaria were refilled to compensate for evaporation and maintain salinity of 36 psu. Aquaria were
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30 92 maintained at 21 ± 1 °C, with a 14:10 h light: dark photoperiod and a light intensity of $100 \mu\text{mol m}^2$
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32 93 s^{-1} . The redox potential of the sand was measured with an Orion ORP 91-80 electrode prior to
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34 94 calibration with a redox buffer solution (220 mV at 25 °C). Measurement of the sediment redox was
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36 95 performed by randomly taking four cores from the sediment-collection area and inserting the
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38 96 electrode to a depth of ca. 4 cm. The sand used as a unconsolidated substrate had a positive redox
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40 97 potential in all pots ($+84.5 \pm 6.9$ mV). Aquaria were aerated to avoid changes in redox potential
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42 98 during the experiment.
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47 99 The lengths of leaves and roots (principals and laterals) were measured after four months.
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49 **Legacy of initial substrate on seedling development and responses to seed-burial**

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51 101 To evaluate the influence of shallow seed-burial and the legacy of substrate hardness, we
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53 102 simulated a restoration event: 60 four-month-old seedlings from the previous experiment were
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55 103 transplanted to sand. Seedlings were carefully removed from the initial substrates by hand, to avoid
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57 104 damaging roots.
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105 We used a factorial design with two fixed factors, *initial substrate* (glass vs. sand) and *seed-*
106 *burial level* (non-buried, half-buried and full-buried). For the “non-buried” treatment, the seedling
107 were anchored on the sand only by their roots, so each seed was totally uncovered and exposed to
108 light. For the “half-buried” treatment, the seed was partially covered (0.5 cm). In the “full-buried”
109 treatment, the seed was covered by sand (1.5 cm) with only the leaves unburied. The sand over the
110 seed reduced light intensity to $1.45 \pm 0.01 \mu\text{mol m}^2 \text{s}^{-1}$ (Fig. 1).

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

116 **Data analysis**

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

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

129 **RESULTS**

130 **Effects of substrate on seedling development**

131 There were significant differences between substrates ($p < 0.001$) in the total length of
132 principal roots and in the number and length of lateral roots. The roots of seedlings germinated on
133 sand (mean = 17.0 ± 5.4 cm) were up to five times longer than those germinated on glass (mean =
134 3.7 ± 1.7 cm; Fig. 1, 2).

135 Total leaf growth had the opposite trend: leaves of seedlings cultured on the glass averaged
136 46.8 ± 7.9 cm per seedling, which was significantly greater than on sand (35.6 ± 10.6 cm per
137 seedling; Fig. 2).

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

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

141 The growth rates of leaves and roots were not influenced by seed size (p-value of the
142 regression = 0.96 and 0.26, respectively). Shadow seed-burial did not affect leaf or root growth rate
143 ($p = 0.39$ and 0.07 , respectively; Table S1). However, seedling development appeared to be
144 influenced by their initial substrate. After the restoration simulation, the growth rates of leaves and
145 lateral roots were significantly higher ($p < 0.01$) in seedlings that had originally developed in sand.
146 In contrast, the growth rate of principal roots was not affected by the initial substrate ($p = 0.71$; Fig.
147 4).

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

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

155 **DISCUSSION**

156 *P. oceanica* showed a high morphological plasticity to two substrates after germination.

157 Sand seemed to promote the growth of principal and lateral roots during the initial four months of

1 158 plant development. Moreover, the use of sand in germinating *P. oceanica* had legacy effects on
2
3 159 subsequent leaf and root development after transplantation to sand. Extensive root growth on sand
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5 160 appeared to be at the expense of leaf development. A similar leaf-root trade-off was observed in *P.*
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7 161 *oceanica* growing on rubble mounds (Di Carlo et al. 2007). However, when seedling were
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9 162 transplanted to sand, all reached same length.

11 163 These results agree with field studies where sand promoted the formation of lateral roots,
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13 164 more than on rock (Balestri et al. 2015). The nutrient content and the unconsolidated structure of the
14
15 165 sand could enhance root growth of *P. oceanica*, while the hard glass limited elongation and nutrient
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17 166 acquisition. *P. oceanica* seedlings have adhesive root hairs that facilitate anchorage on rocky
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19 167 substrates (Badalamenti et al. 2015). Likewise, roots on glass were fully adhered to the surface in
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21 168 our experiment. *Phyllospadix scouleri* Hook. also has root hairs that help anchor plants to substrates
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23 169 in intertidal zones (Short & Coles 2001; Kuo & Den Hartog 2006).

25 170 *P. oceanica* seeds photosynthesize even after germination (Celdrán & Marín 2011), so it is
26
27 171 possible that shallow seed-burial could negatively affect development. Nevertheless, growth of
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29 172 four-month-old seedlings was not influenced by seed-burial. It is likely that the leaves of young *P.*
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31 173 *oceanica* soon compensate for any reduced photosynthesis by seeds.

33 174 The general procedure for restoration of *P. oceanica* is to attach transplants to the substrate
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35 175 using non-biodegradable materials (Augier et al. 1996; Balestri et al. 1998, 2011; Meinesz et al.
36
37 176 1992; Meinesz et al. 1993; Molenaar et al. 1993; Molenaar & Meinesz 1992). A well-developed
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39 177 root system is expected to maximize anchoring capacity and nutrient acquisition (Balestri &
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41 178 Lardicci 2005; Schutten et al. 2005; Statton et al. 2014). Infantes et al. (2011) evaluated the
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43 179 substrate-anchoring capacity of *P. oceanica* seedlings for the hydrodynamic conditions of the
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45 180 Mediterranean and estimated the minimal root length needed by seedlings to prevent dislodgement
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47 181 from sand (0.35 times the square root of the leaf area). Using this equation, 100% of our seedlings
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49 182 on sand and 13% of those on glass would remain attached to a sandy seabed.

51 183 Germination of *P. oceanica* seedlings on sand enhanced the growth of principal root and had
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53 184 a legacy of enhanced leaf and lateral root growth after transplantation. However, shallow burial of
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1 185 the seed did not slow the development of five-month-old *P. oceanica* seedlings. Thus, we suggest
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3 186 culturing seedlings on sand before transplantation, to facilitate anchorage and avoid using non-
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5 187 biodegradable materials in seagrass restoration. Transplantation to sand should be limited to periods
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7 188 of calm weather and in bays that are protected from strong hydrodynamic events.
8

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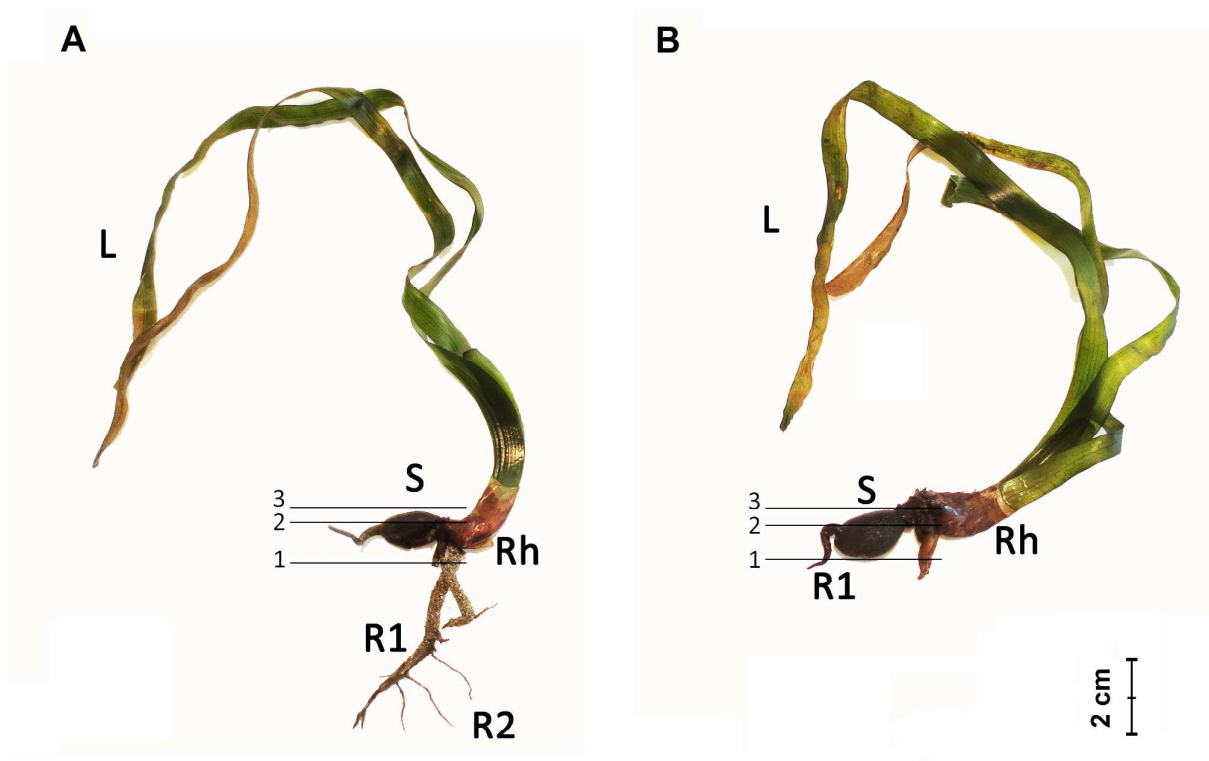
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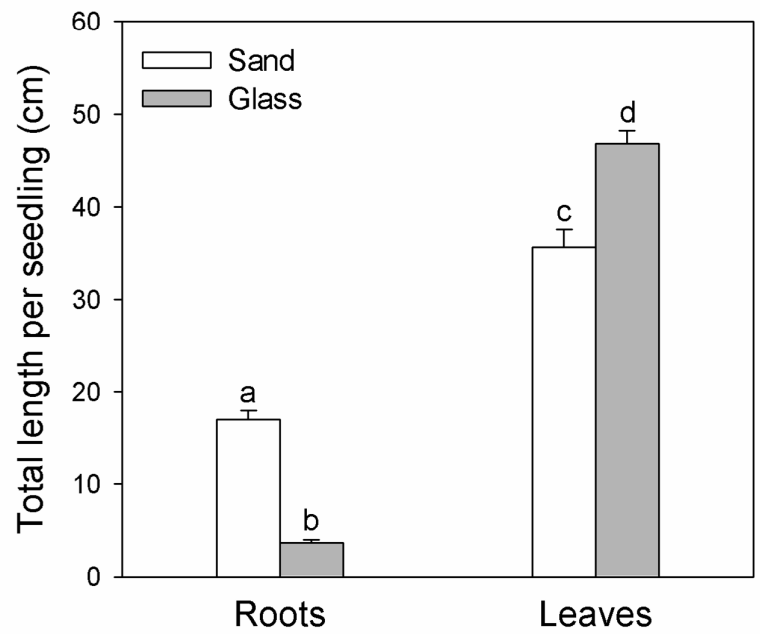
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33 308 *Geology* 30:377–392
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35 309 **ARTWORK AND TABLES WITH CAPTION:**
36
37 310 **Figure 1.** *P. oceanica* seedlings germinated in sand (A) and glass (B). Numbers indicate the shadow
38
39 311 seed-burial level used in the restoration simulation: 1: non-buried; 2: half-buried; 3: full-buried. L:
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41 312 leaves; S: seed; Rh: rhizome, R1: principal roots; R2: lateral roots.
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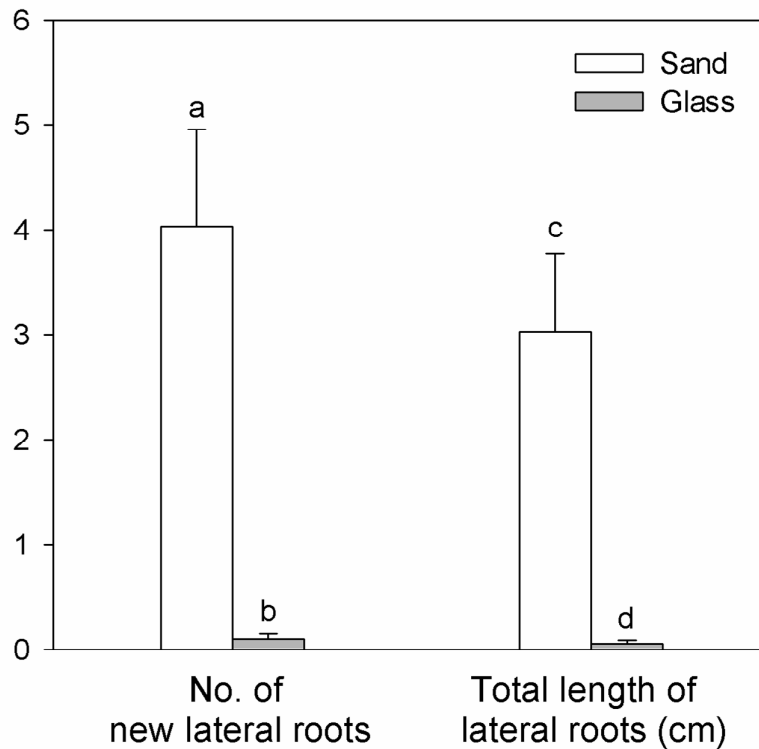
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314 **Figure 2.** Growth of principal roots and leaves per seedling germinated in sand and glass (mean +
 315 SE; n=30). Letters above the bars indicate significant differences between substrate types (Student's
 316 *t* - test, $p < 0.05$).



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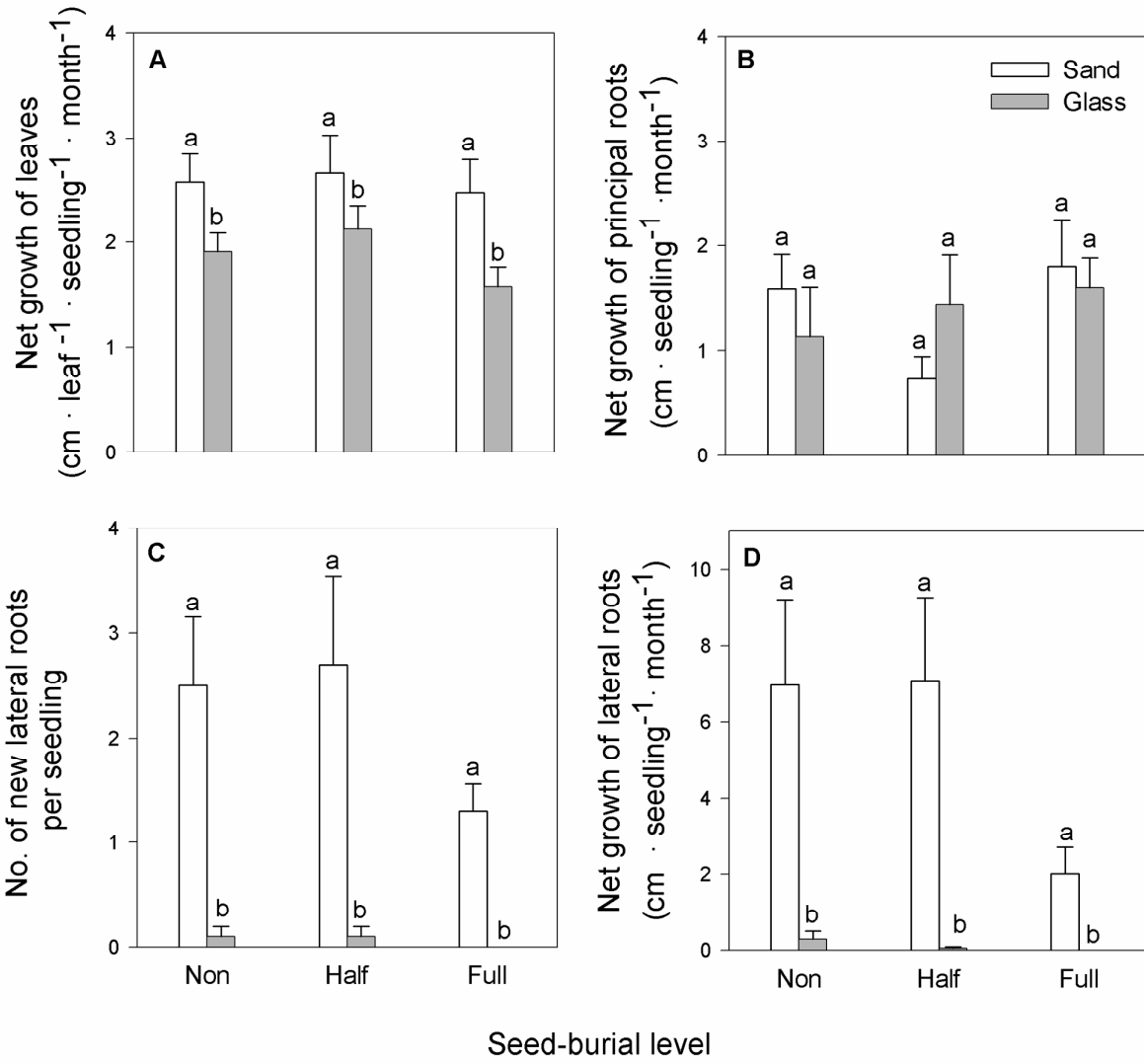
318 **Figure 3.** Number of new lateral roots and total length of lateral roots per seedling germinated in
 319 sand and glass (mean + SE; n=30). Letters above the bars indicate significant differences between
 320 treatments (Student's *t*-test, $p < 0.05$).



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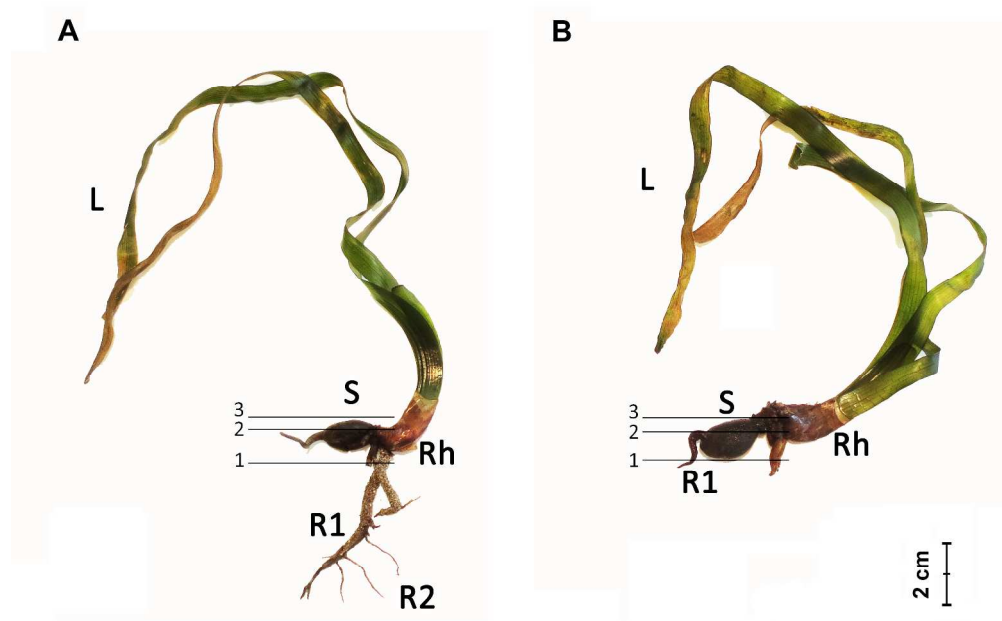
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323 **Figure 4.** Growth of leaves (A), growth of principal roots (B), number of new lateral roots (C) and
 324 growth of lateral roots per seedling (D) after of the restoration simulation for the treatments *Seed-*
 325 *burial level* (non-buried,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



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Review



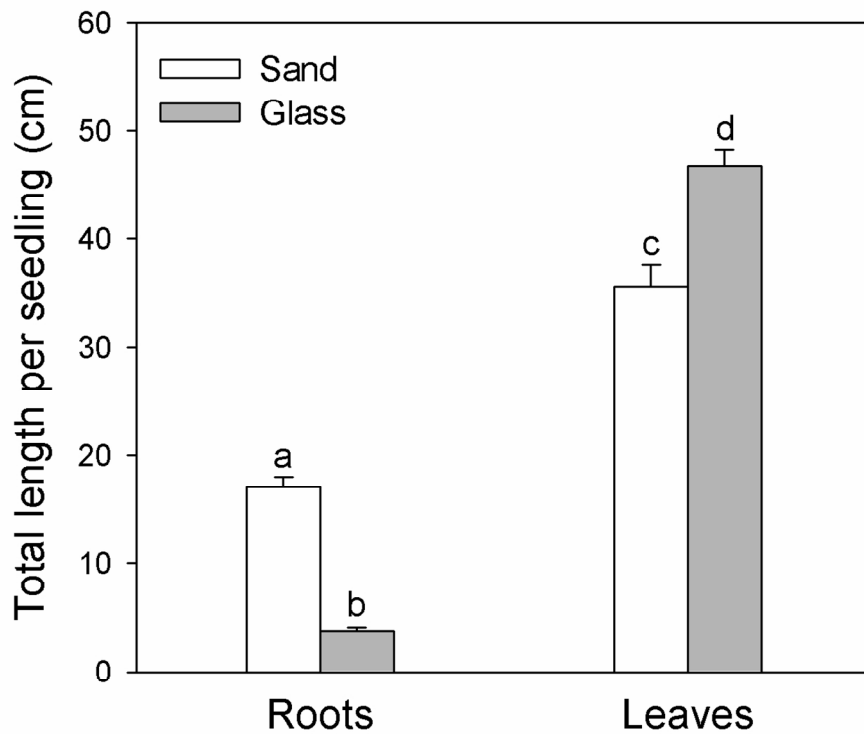
P. oceanica seedlings germinated in sand (A) and glass (B). Numbers indicate the shadow seed-burial level used in the restoration simulation: 1: non-buried; 2: half-buried; 3: full-buried. L: leaves; S: seed; Rh: rhizome, R1: principal roots; R2: lateral roots.

289x194mm (300 x 300 DPI)

review

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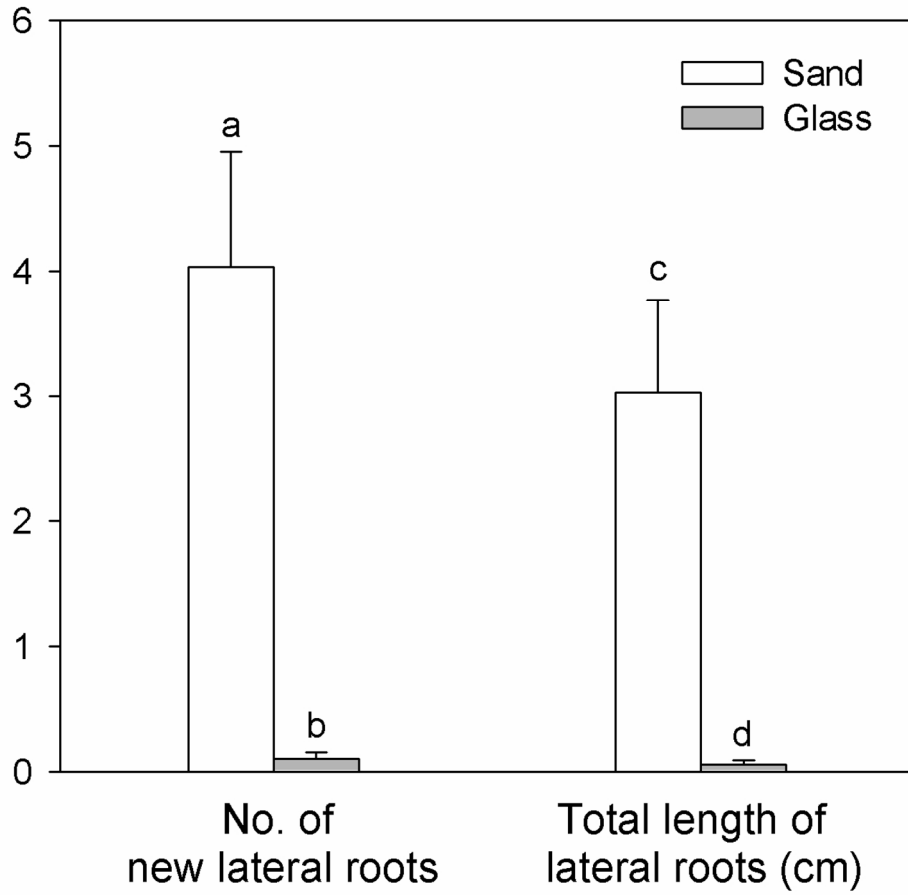


Growth of principal roots and leaves per seedling germinated in sand and glass (mean + SE; n=30). Letters above the bars indicate significant differences between substrate types (Student's t - test, p< 0.05).

120x104mm (300 x 300 DPI)

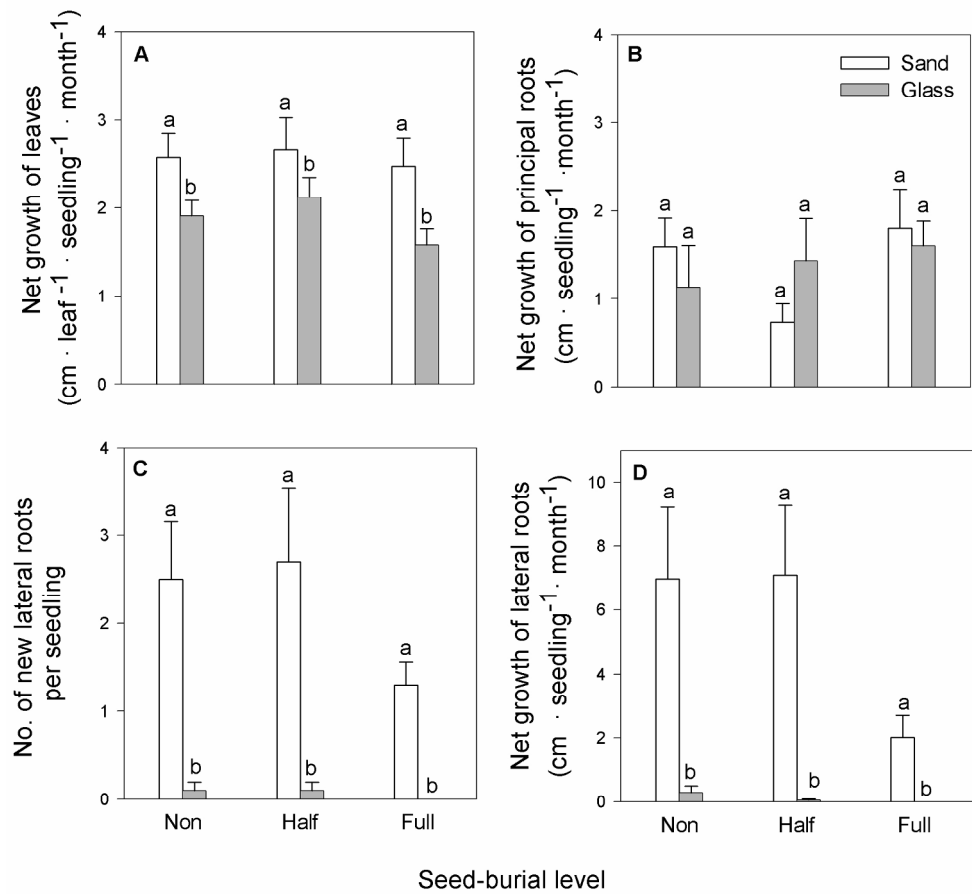


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Number of new lateral roots and total length of lateral roots per seedling germinated in sand and glass (mean + SE; n=30). Letters above the bars indicate significant differences between treatments (Student's t-test, $p < 0.05$).

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)



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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

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6 Levene's test: $p = 0.00$
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8 Transformation: $\text{Ln}(x + 1)$
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11 **New lateral roots per seedling**

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13 Initial Substrate (S) 1 12.14 60.29 < **0.001**
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15 Seed-Burial level (B) 2 0.33 1.64 0.20
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17 S x B 2 0.08 0.38 0.69
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19 Residual 54 0.20
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23 Levene's test: $p = 0.00$
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25 Transformation: $\text{Ln}(x + 1)$
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