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Restoration of *Cymodocea nodosa* seagrass meadows through seed propagation: germination *in vitro*, seedling culture and field transplants

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Abstract

Cymodocea nodosa, a marine angiosperm, an ecosystem engineer in the Mediterranean Sea and the Northwest Atlantic Ocean; however, as in other seagrasses meadows worldwide, the swards are actually declining due to increasing human pressures. Hence, we have developed an effective propagation methodology that provides *C. nodosa* seedlings for seagrass meadow restoration and conservation. This method consists of: i) germination of wild-collected seeds under hyposaline conditions, ii) acclimation of germinated seedling in tanks (1.6 m^3) until there are two shoots per seedling (~30 days), and iii) transplantation of acclimated seedlings to the field in dense groups. Our field outplants withstood herbivore activity and physical disturbance during the winter season, and propagated vegetatively, resulting in the spread and establishment of a new patch that has persisted for nine months.

Keywords: acclimation; germination; hyposalinity; restoration; seagrasses.

Introduction

Concern has arisen over the decline of seagrass ecosystems worldwide as a result of adverse human activities in coastal areas (Walker and McComb 1992, Duarte et al. 2004, Orth et al. 2006, Duarte and Gattuso 2008, Hughes et al. 2009). Restoration and mitigation programs have been carried out since the mid-20th century (Addy 1947, Phillips 1974, Gordon 1996, Fonseca et al. 1998, Johansson and Greening 2000) using plant material collected from donor meadows (Orth et al. 1999, Heidelbaugh et al. 2000, Van Keulen et al. 2003, Paling et al. 2007, Park and Lee 2007). Because of the logistical burden associated with moving mature plants, the use of seeds and seedlings germinated *in vitro* is widely considered to be a more cost-effective alternative (Thorhaug 1985, Orth et al. 1994, 2000, 2009, Balestri et al. 1998, Kirkman 1998, Harwell and Orth 1999, Granger et al. 2002, Pickerell et al. 2005, Ailstock and Shafer 2006). This method is also likely to avoid damage to donor meadows from which planting units (comprising adult plants) are extracted (Christensen et al. 2004, Seddon 2004); transplanting seeds and seedlings also increases genetic variability in the receiving population that is to be restored or replanted (Waycott 1995, Williams and Orth 1998, Procaccini and Piazzi 2001, Reusch 2002).

The seagrass *Cymodocea nodosa* (Uchria) Ascherson forms dense, shallow water meadows (between 2 and 35 m deep) across the Mediterranean Sea and the Northwest Atlantic Ocean, including the Canary Islands (Reyes et al. 1995, Pavón-Salas et al. 2000, Alberto et al. 2008, Cunha and Araújo 2009). This marine angiosperm produces permanent seed banks throughout the year (Caye and Meinesz 1985, Caye et al. 1992, Terrados 1993, Reyes et al. 1995), but germination rates in nature may be variable. Pirc et al. (1986) and Reyes et al. (1995) reported germination rates as high as 50%, but sometimes the rate is zero (Caye and Meinesz 1985); moreover, once seeds have germinated, their fate is mixed, and actual recruitment may be low (Caye and Meinesz 1986, Buia and Mazzella 1991, Terrados 1993, Reyes et al. 1995).

The sensitivity of this species to human impacts has been documented in several studies (Terrados and Ross 1995, Vergara-Martín et al. 2005, Fernández-Torquemada and Sánchez-Lizaso 2006), and a restoration protocol for conservation is urgently required. To this end, two studies have been performed to transplant *Cymodocea nodosa* adult plants from donor to recipient meadows (Curiel et al. 2003, Ruiz de la Rosa et al. 2006), but there have been no attempts to transplant seedlings.

Given the fragility of *Cymodocea nodosa* in the face of human impacts, and its low germination and seedling survival rates in nature (Caye and Meinesz 1985, Buia and Mazzella 1991), we believe that increasing germination potential and seedling survival would be an appropriate strategy for the conservation of this species and its habitat. In this study, we developed a methodology for propagation of *C. nodosa* seeds under laboratory conditions, the acclimation of seedlings to enhance biomass development, and outplanting in the wild under prevailing conditions ensuring enhanced survival rates. This methodology provides seedlings that might be employed for the restoration of small but strategically important degraded areas, or to improve the conservation of established meadows by introducing genetic variation.

Materials and methods

Seed collection and culture methods

The seeds used in this study were collected by SCUBA diving (May 2007) in a *Cymodocea nodosa* meadow located off Juan Grande on the southeastern coast of Gran Canaria (27°45′45.48″ N; 15°33′04.01″ W, Spain). Seeds were found either on the surface of the seafloor or buried a few cm beneath the sediment. Once collected, seeds were placed in a nylon net (0.2-mm mesh), which kept them wet during transfer to the laboratory (within 2 h of collection).

To prevent overgrowth by epiphytes, seeds were gently cleaned with a tooth brush and then submerged for 10 min in a 10% (v:v) commercial bleach solution made up in autoclaved seawater (a drop of Tween 80 was used as surfactant). Seeds were then washed three times in autoclaved seawater. Culture vessels (Magenta^R – G7 300 ml Sigma Co., Chicago, IL, USA) were filled with 40 cc of autoclaved sand and 200 ml of liquid culture medium. We used PES culture medium (Provasoli 1968), but substituted ammonium for nitrate and mono-potassium phosphate for glycerol phosphate, based on previous experience with *Cymodocea nodosa* explant cultures (see García-Jiménez et al. 2006). The salinities of the seawater and culture medium in culture matched ambient seawater (36.6 psu), except where otherwise stated.

Cultures in the laboratory were placed inside a growth chamber at 24°C, with a 16:8 light:dark photoperiod and 30 μmol photons m^{-2} s^{-1} irradiance at the level of the culture vessels.

Induction of germination

Hyposaline shock was used to induce germination. This treatment facilitates the imbibition of water needed for seed germination (Caye and Meinesz 1986, Caye et al. 1992). Seawater diluted with double distilled water was used to achieve the different salinity levels: 5, 11 and 18 psu, while 36 psu was used as a control treatment (Robaina et al. 1990a,b). Thirteen culture vessels with two seeds in each (i.e., 26 seeds per treatment) were used for each treatment to determine the effects of hyposaline treatments on germination. Thirty days (based on preliminary experiments) after hyposalinity shock, all seeds and seedlings were transferred to 36.6 psu medium to acclimate them to ambient salinity, and three different stages of development were monitored: stage I, germinating seeds (Figure 1A); stage II, stage with emergent green cotyledon (Figure 1B), and stage III, development of first new leaves (Figure 1C). These three stages of development extended over three months following germination induction.

Acclimation of seedlings

All viable seedlings that had reached stage III after the induction procedure were placed into holding tanks (1.6 m^3) with sand covering the bottom (ca. 10% of the total volume, 15 cm depth, no nutrients added) under open, continuous seawater flow, at an average temperature of 21°C and an average irradiance of 60 μmol photons m^{-2} s^{-1} (Figure 1D). Leaf and root growth and shoot and root emergence were monitored fortnightly (n=30) while plants were in the holding tanks (45 days) prior to transplantation to the field.

Transplants of developed seedlings to the field

Once plants had developed sufficient roots and shoots for transplantion (30–45 days), they were transferred to the field. Two pilot transplant treatments (May 2008) were compared: i) seedlings scattered on nylon nets tied with thread (20×20 cm nets with 20 sown seedlings in each, n=60, density=500 seedlings m^{-2}), and ii) seedlings clustered in biodegradable trays made of compressed coconut fiber dust (trays hereafter), which allowed a healthy root growth in 12 compartments of 3×3×5 cm, with three seedlings per compartment and up to 36 seedlings per tray, n=108, density=3333 seedlings m^{-2}). A set of seedlings (n=76) was planted directly in the sediment; (density=3377 seedlings m^{-2}). The nylon nets and trays were anchored using metal staples. For these pilot transplanting experiments, a sheltered location (6 m depth, along the edges of a natural meadow, 27°59′20.44″ N; 15°22′12.45″ W, Gran Canaria, Spain) was selected to improve chances of establishment and for monitoring purposes. Over nine months, we monitored % survival (as % seedlings remaining in the patch and apparently pigmented and healthy) and average leaf length (cm) for each transplant methodology and for clustered seedlings that had been planted directly in the sediment.

Statistics

Statistical design and calculations were carried out using SPSS® 13.0 for Windows® (SPSS Inc., Chicago, IL, USA). Contingency table analyses were used to detect differences on germination and further growth in 18 psu salinity and the rest of the treatments, assuming H_0:p1, proportion in 18 psu=p2, proportion in other salinity treatments, and the alternative hypothesis was: $p1 \neq p2$; the test statistic was χ^2. Bonferroni correction to the experiment-wise error rate (5%) was applied to reduce the type 1 error rate in a set of multiple comparisons. In the outplanting experiment, ANOVA was used to compare the number of seedlings remaining each month between trays and nylon nets.

Results

We found an inverse relationship between culture medium salinity and seed germination (Table 1). The lower the salinity, the greater the germination rate. However, responses in treatments 5, 11 and 18 psu were not significantly different at the end of the experiment; all salinities induced seed germination within a month. Significantly lower germination occurred in the control treatment (36 psu). Regardless of treatment, 60% of cultivated seeds germinated after three months (in 82.6% of culture vessels).

The reduced salinity treatments inhibited post-germination development (Table 1). None of the seeds germinated at

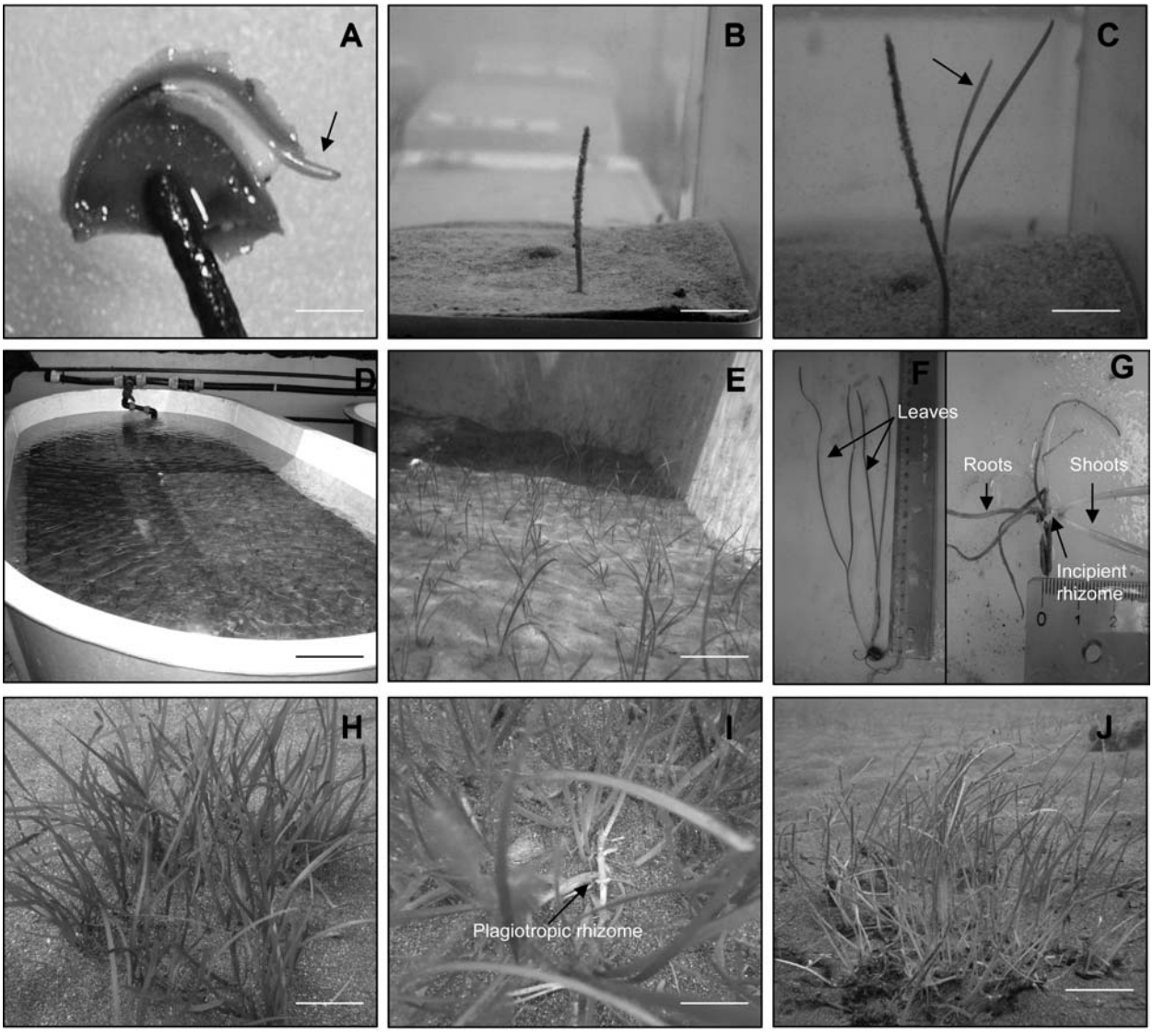

Figure 1 *Cymodocea nodosa.*
(A–C) Three different stages of development: (A) Stage I, germinating seeds (arrow), bar=0.5 cm; (B) Stage II, elongation of cotyledon, bar=1 cm; (C) Stage III, development of first new leaves (arrow), bar=1 cm; (D–G) Acclimation: (D–E) Seedlings acclimating in aquaria or tanks; (D) bar=0.5 m; (E) bar=0.4 m; (F–G) Seedlings with leaves attached to a young incipient rhizome with up to two leaves and root development after 30 days of acclimation; (H–J) Field transplant. Persistent patch formed from clustered seedlings transferred directly into the sediment: (H) Plants at day zero, bar=5 cm; (I) Plants at day 30. Note plagiotropic rhizome development, bar=4 cm; (J) Plants at month 6, bar=5 cm.

5 psu reached stage II and III *in vitro* (Figure 1B, C). Considering both germination percentage and further development up to stage III, the best germination and seedling development was achieved at salinities of 11 and 18 psu (slightly higher in the latter), as the seeds apparently acclimated *in vitro* and even generated two new leaves (Figure 1C).

All seeds that reached stage III developed well after transfer to continuous seawater-flow tanks (Figure 1D, E), regardless of whether or not they had germinated in media at 11, 18 or 36 psu. Seedlings grew and developed new roots, shoots and leaves during the whole acclimation period (Figure 2A, D). At day 30, seedlings had developed 4.20±0.95 leaves, 1.40±0.50 shoots and 4.05±1.19 roots, with average leaf and root lengths of 22.25±6.45 cm and 7.46±2.90 cm, respectively (Figure 2A–D).

After outplanting to the sea, there were no significant differences between the number of seeds surviving in trays and nylon nets until month five (Figure 3A). Thereafter, seeds attached to nets died and only few necrotic fragments remained attached, while seedlings in the trays remained (ca. 40% survival). The best results were obtained by direct transplantation into the substratum (Figure 3A). These seedlings survived a winter and are now almost a year old, forming a small patch in which new plagiotropic rhizome tissue was observed (Figure 1H–J). Leaf length was very varied when

Table 1 *Cymodocea nodosa*: effect of salinity treatments on germination and further growth to reach development stage III (3 months).

	Month 1				Month 2				Month 3			
Salinity (psu)	5	11	18	36	5	11	18	36	5	11	18	36
Germination (n)	12*	12*	7	–	12	12	12	5**	12	12	13	6**
Germination (%)	92.3	92.3	53.84	–	92.3	92.3	92.3	38.46	92.3	92.3	100	46.15
Stage III (n)	–	1	1	–	–	7	11	5**	–	9	12	6**
Stage III (%)	–	7.69	7.69	–	–	53.84	84.61	38.46	–	69.23	92.3	23.07

Percentage germination: number of culture vessels in which germinating seeds were observed among total number of culture vessels (13 vessels per treatment; total seeds=104). Percentage stage III: number of vessels in which germinated seeds reached stage III (i.e., seedlings with one shoot and two leaves). Experiment-wise error rate is 0.05. Comparison error rate is experiment-wise error rate/number of comparisons (Bonferroni correction). *, ** Indicates significant differences between a treatment and 18 psu treatment in each month ($p<0.017$ and $p<0.01$, respectively; –, not detected).

seedlings were transplanted into the sea, but finally stabilized at the end of the experiment (Figure 3B).

Discussion

The importance of seagrass beds has been recognised worldwide for their multiple ecological roles in estuarine and coastal systems, where they act as nursery habitat and enhance coastal erosion protection (Mena et al. 1993, Terrados and Borum 2004). However, estuaries and coasts and the species that inhabit them are vulnerable to increasing human pressure (den Hartog 1996, Orth et al. 2006, Ralph et al. 2006, Hughes et al. 2009) and habitat conditions have substantially deteriorated over the last two decades (Duarte et al. 2004). Application of diverse management strategies

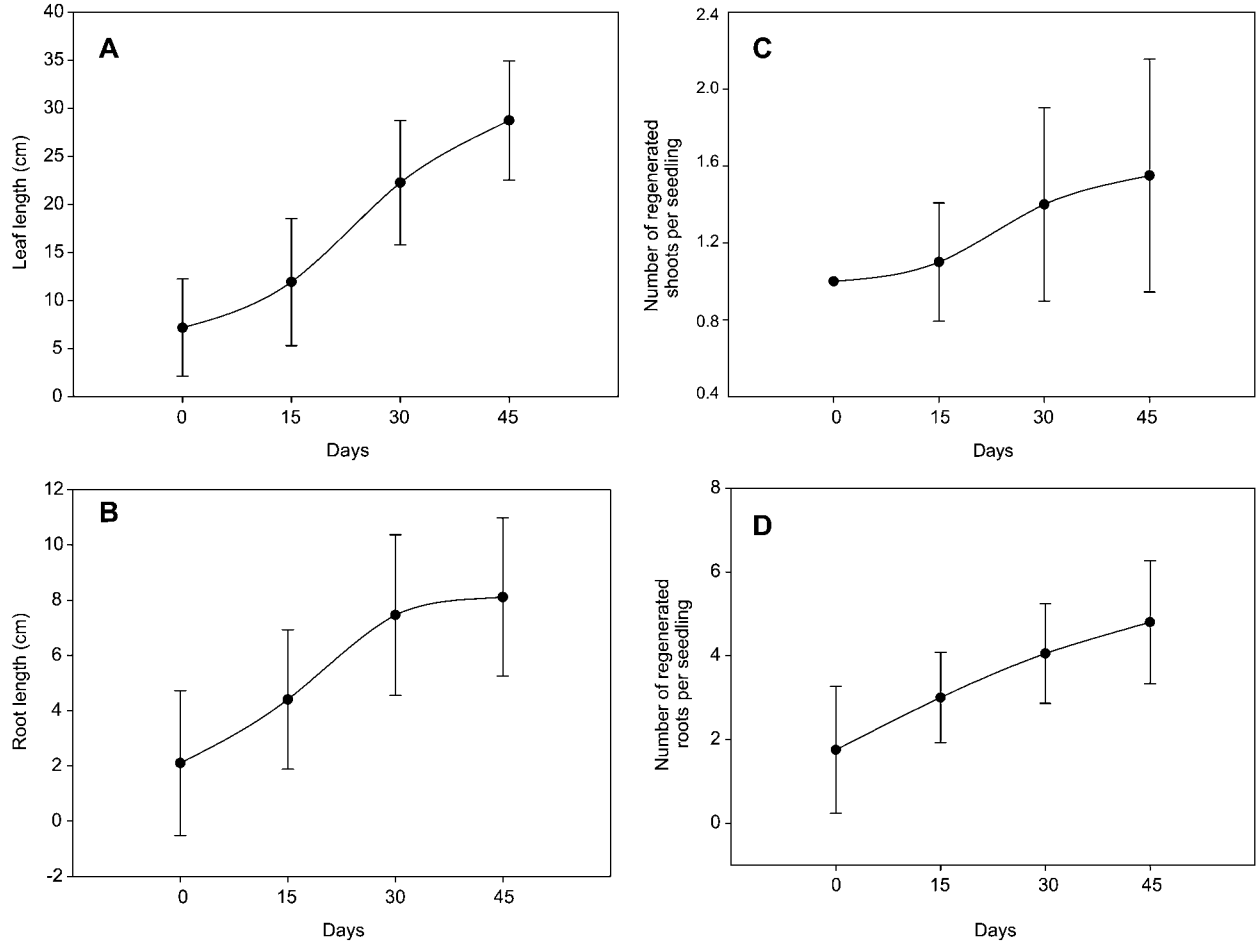

Figure 2 *Cymodocea nodosa*: plant development during acclimation.
(A–B) Leaf and root lengths over 45 days. (C–D) Shoot and root regeneration over 45 days seedling acclimation (values are means±SD, n=30).

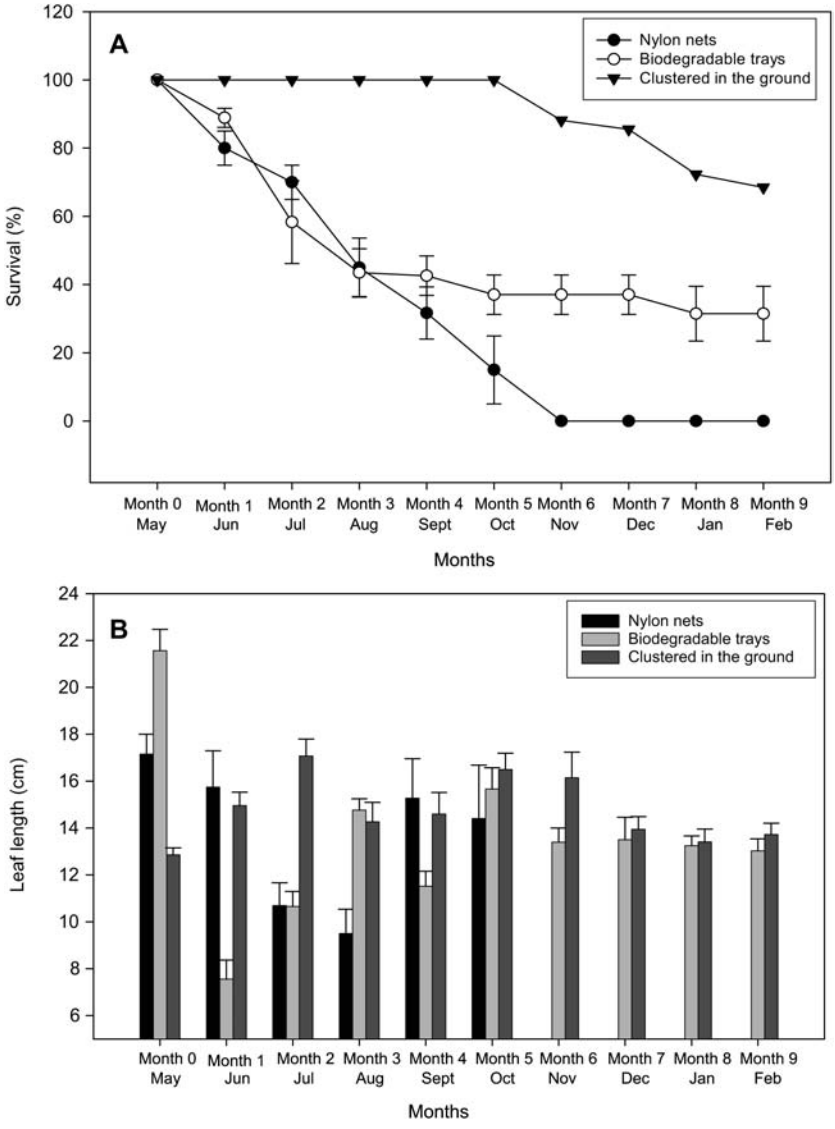

Figure 3 *Cymodocea nodosa:* field trials.
(A) Survival (%). (B) Average leaf length (cm) of acclimated plantlets transferred to a natural meadow (27°59′20.44″ N; 15°22′12.45″ W, Gran Canaria, Spain) using three different transplant methodologies: a) seedlings scattered on nylon nets (r=3, n=60), b) seedlings clustered in biodegradable trays (r=3, n=108), and c) seedlings planted directly into the sediment (n=76). Seedling monitoring was performed over nine months (values are means±SE).

for the conservation of these ecosystems is a pressing requirement (Erftemeijer et al. 2006). In Tampa Bay (Florida), control of water quality in *Thalassia testudinum* Banks *ex* Konig habitats allowed recuperation of meadows, which increased in area from 8800 ha in 1982 to 10,930 ha in 1997 (Johansson and Greening 2000). Rehabilitation, restoration and mitigation programs have been carried out worldwide with different species and with varying degrees of success (Lord et al. 1999, Fonseca et al. 2000, Van Katwijk 2003, Seddon 2004, Paling et al. 2007). As for other seagrass species, *Cymodocea nodosa* is affected by human impacts (Vergara-Martín et al. 2005, Fernández-Torquemada and Sánchez-Lizaso 2006), and some efforts have been made to protect and conserve them. At the present time, *C. nodosa* is included in the Catalogue of Endangered Canarian Species (BOC 2001, decree 151/2001), and their habitat is protected by the EU in the Habitats Directive (Council Directive 92/43/EEC).

In this work, we have developed a propagation protocol for *Cymodocea nodosa* seeds that may be useful for restoration and conservation programs. The procedure includes i) germination of seeds *in vitro*, ii) acclimation and further growth of seedlings in tanks, and iii) their later transference to coastal marine habitats.

Propagation protocol for *Cymodocea nodosa*

Germination of collected seeds under hyposaline treatments In *Cymodocea nodosa* from the Canary Islands, optimal seed germination occurred at 50% of ambi-

ent salinity (36 psu), although equivalent effects may be obtained with salinity as low as 11 psu. Our results are congruent with other seagrass studies that have reported hyposaline conditions to be favorable for the germination of seeds (Caye and Meinez 1986 and Caye et al. 1992 for *C. nodosa*; Hootsmans et al. 1987 for *Zostera marina* L. and for *Z. noltii* Hornem.; Harrison 1991 for *Z. marina*; Conacher et al. 1994 for *Z. capricorni* Aschers.; Ailstock and Shafer 2006 for *Ruppia maritima* L. and for *Potamogeton perfoliatus* L.; Alexandre et al. 2006 for *Z. marina*).

The number of germinated seeds eventually produced through exposure to hyposaline shock (5, 11, 18 psu) was significantly higher than that in non-shocked treatments (36 psu). However, it is important to note that the highest germination rates did not necessarily provide the highest number of seedlings for further propagation. At 5 psu, *Cymodocea nodosa* seeds had the highest percentage germination in the first month, but further development of the seedlings was restricted at this low salinity (Table 1). There may be an optimal hyposaline treatment for each species that should be determined before proceeding. Moreover, temperature, light or even chemical treatments should not be disregarded as effective methods to break seed dormancy (Hilhorst and Karssen 1992). Considering both germination and seedling viability after hyposaline treatment, 11 and 18 psu (slightly better at the higher salinity) appeared to be suitable for further seedling propagation. The germination of seeds in enriched seawater (modified PES) and 50% of normal salinity, with further salinity increases to 36 psu after 30 days in culture, supported a transition rate of 54% from germination to stage III of development (92.3% germinated individuals reached stage III of development, Figure 1C).

Acclimation of germinated seeds in tanks Acclimation is considered a mandatory step when propagating terrestrial plants using *in vitro* techniques, since plants derived from cell cultures under controlled conditions may be shocked when transferred directly to the field (Pospíšilová et al. 1999). The acclimation of seagrass seedlings to optimize transplantation survival and growth has been emphasized in previous work (Meinesz et al. 1993, Bird et al. 1994, Woodhead and Bird 1998). However, it is also important to use the minimum feasible acclimation-period to make the protocol more cost-effective. For this reason, we consider a 30-day period sufficient to produce healthy *Cymodocea nodosa* seedlings (100% of germinated seed) ready for transplantion to the natural environment (Figure 1F). By this time, we had produced seedlings with up to four leaves (>20 cm long) and substantial root development (up to four single roots and 7 cm long) (Figures 1F, G and 2).

Even though previous authors have germinated *Cymodocea nodosa* seeds under laboratory conditions (Caye and Meinesz 1986, Caye et al. 1992), further growth in these earlier works was limited, with seedling death after three to four months (Pirc et al. 1986, Buia and Mazzella 1991). The propagation systems used in our work (see Materials and methods) allow germination and continuous growth of seedlings, producing healthy plants ready for transplant in a four-month period.

Field transplants Use of seeds and seedlings in restoration programs has been considered as a cost effective technique that could introduce genetic variability into damaged meadows (Orth et al. 2000, Procaccini and Piazzi 2001, Ailstock and Shafer 2006). Previous works on seedling transplantation have experienced problems with anchoring, and different attachment protocols have been developed to settle seedlings into the sediment (Meinesz et al. 1993, Kirkman 1998, Woodhead and Bird 1998, Seddon et al. 2004, Wear et al. 2006). Perhaps unexpectedly, our results suggest that anchoring of seedlings and plantlets might not be so critical for *Cymodocea nodosa* when seedlings are transplanted to a sheltered place without any anchoring method, as seen for seedlings directly planted into the sediment (Figure 3A). These seedlings developed plagiotropic rhizomes one month after transplantation (Figure 1I). According to Kirkman (1998) and Marbá et al. (2004), the development of plagiotropic rizhomes signifies the establishment of vegetative clonal growth necessary for the formation of new patches. This predicts great success for the future survival of transplanted seedlings; Marbá et al. (2004) reported that 50–70% of seagrass seedlings die in nature before clonal growth begins. Furthermore, clonal growth allows the accumulation of carbon and mineral nutrients to maintain growth in unfavorable seasons and for resource allocation in the face of tissue loss to grazing (Vergés et al. 2008).

Our field observations (i.e., bites on the leaf) pointed to herbivory as a possible explanation for the highly variable leaf lengths observed in transplanted seedlings (Figure 3B). We believe that clustered seedlings in the field and the resulting development of plagiotropic rhizome may also have mitigated herbivore attack, as was the case for *Posidonia* sp. in Western Australia (Kirkman 1998). This may help explain higher success when seedlings were clustered at an initial density of $\sim$3000 s m^{-2} (i.e., in trays and direct transplanting methods, Figure 3A).

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