Seed maturity of the Mediterranean seagrass Cymodocea nodosa

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SEED MATURITY OF THE MEDITERRANEAN SEAGRASS *CYMODOCEA NODOSA*

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CYMODOCEA NODOSA
SEEDS
TIME OF COLLECTION
INDUCTION OF GERMINATION
SEEDLINGS
VEGETATIVE DEVELOPMENT

ABSTRACT. – The germination of seeds in aquaria and posterior planting in the sea is an approach that may be applied in seagrass restoration projects. Hypo-salinity is known to induce germination of *Cymodocea nodosa* but the appropriate period for seed collection is useful information that has not been considered until now. The aim of this study was to evaluate the maturity of *C. nodosa* seeds by comparing the effectiveness of the hypo-salinity induction of germination in seeds collected in August, September and February and detect possible differences in the development of the seedlings. None of the seeds collected in August germinated after hypo-salinity induction and died during the following months of storage in aquaria. The September and February seeds showed high germination percentage after hypo-salinity induction (> 90 %) and most of them develop into seedlings that showed similar vegetative development. Germination of seeds under full salinity was much slower and scarce (less than 15 % after six months). It was feasible to produce well-developed seedlings of *C. nodosa* with 3-4 leaves and 2 roots within five weeks from seeds collected from September to February.

INTRODUCTION

Seagrass meadows play important roles in coastal processes because they enhance biological productivity and biodiversity, stabilize the sediment and reduce coastal erosion, and trap suspended particles and dissolved nutrients in the water column (Hemminga & Duarte 2000). These ecosystems are highly sensitive to water quality degradation and seagrass meadows are one of the most threatened coastal ecosystems (Orth *et al*. 2006, Waycott *et al*. 2009). The renewed interest in seagrass restoration during the last decade (Christensen *et al*. 2004, Orth *et al*. 2006), increased the need for seagrass research, from the production of new knowledge about seagrass biology and ecology relevant to restoration (Kenworthy *et al*. 2006) to the adaptation of existing restoration techniques to local species and conditions (Calumpong & Fonseca 2001).

Cymodocea nodosa (Ucria) Ascherson is the second most important seagrass concerning the surface area covered in the Mediterranean after *Posidonia oceanica* (L.) Delile (Procaccini *et al*. 2003). Like in most seagrasses, clonal growth is the species main mechanism of proliferation, but in restoration projects the use of seeds or seedlings is considered to be more advantageous as it reduces the damages to donor meadows, facilitates the logistics involved in the production and handling of planting units, and assures a certain level of genetic diversity of the planted populations (Fonseca *et al*. 1998, Procaccini & Piazzi 2001, Christensen *et al*. 2004).

Cymodocea nodosa produces flowers and fruits yearly, and seed banks are common (Caye & Meinesz 1985, Terrados 1993, Buia & Mazzella 1991, Cancemi *et al*. 2002). The experiments performed by Caye & Meinesz (1986) showed that it is possible to induce the germination of *C. nodosa* seeds in the laboratory using a hypo-saline treatment (germination might occur in 2-6 days), but the state of maturity of the fruits and, consequently, the appropriate period for their collection is relevant information that has not been considered until now. The aim of this study was to evaluate the maturity of *C. nodosa* fruits by comparing the capacity of fruits collected in different months to develop into seedlings.

The hypo-saline induction of *C. nodosa* seeds may allow obtaining high germination percentages in short time and a sufficiently high number of seedlings for restoration. Here we investigated the feasibility of producing seedlings of *Cymodocea nodosa* from seeds collected at different times along the year considering that seed maturity may change with time. *C. nodosa* flowers from April to June, the fruits develop from July to October (Caye & Meinesz 1985, Terrados 1993, Buia & Mazzella 1991, Cancemi *et al*. 2002), and the seeds start to germinate in April or May of the following year (Pirc *et al*. 1986, Buia & Mazzella 1991). Seeds were collected in August 2008, when fruits are attached to the shoot, in September 2008, when fruits are still attached to the shoot but fruit pericarp starts to degrade, and in February 2009, when all the fruits had lost the pericarp and had become loose seeds in the sediment. A hypo-saline induction (Caye & Meinesz 1986) of seed germination was used to assess if the seeds were viable and sufficient mature to produce seedlings. The early vegetative development of the seedlings produced was compared.

METHODS

The fruits/seeds of *Cymodocea nodosa* were collected by SCUBA diving between depths of 2-3 m inside a patchy meadow located in the Bay of Pollença, North coast of Mallorca, Balearic Islands, Spain (N 39º 51' 41.97"; W 3º 6' 47.46"). The sampling was carried out in August 2008, in September 2008 and in February 2009, resulting in the collection of 79, 330 and 233 fruits/seeds, respectively. We consider that all the fruits/seeds collected were produced during the summer of 2008, as no loose seeds were found in the sediment in August 2008 and September 2008.

The fruits/seeds were transported to the laboratory in aerated seawater and ambient temperature within 3 hours of collection. They were maintained until the beginning of the experiment in February 2009, inside a temperature-controlled room in 60 L aquaria over a layer of sediment (fine sand) of the collection site, at full salinity (36-37) seawater, 23 ºC, and a light:dark photoperiod of 14:10 h.

Eighteen 20 L aquaria were set inside the temperature-controlled room and each was equipped with a filter, an air pump and a 9W white-light fluorescent lamp (Philips MASTER PL-S9W/840/222P) that provided a photosynthetically active photon flux density of 17.5 ± 4.2 μmol m^{-2} s^{-1}. Half of the aquaria was filled with full salinity (36-37) seawater and the other half was filled with seawater mixed with distilled water to achieve a salinity of 10. This hypo-saline treatment induces germination in less than a week (Caye & Meinesz 1986). Salinity was controlled regularly with an YSI Model 30 handheld salinity probe. No sediment was placed in the aquaria to assure the constancy of the salinity treatment of the fruits/seeds. The fruits/seeds from each month of collection were distributed in 6 of the 20 L aquaria, 3 with full salinity seawater and 3 with a salinity of 10. The number of fruits/seeds per aquaria was 13 for the August 2008 collection, 55 for the September 2008 collection, and 39 for the February 2009 collection. The aquaria were kept at a temperature 20-21 ºC, which allows a seed germination rate above 90 % within two weeks and a good development of the seedlings (Caye & Meinesz 1986).

Seeds were considered germinated when the dorsal ridge of the endocarp opened and began to show the cotyledon (Fig. 1A). The hypo-saline treatment was maintained for 15 days. Caye & Meinesz (1986) showed that germinated seeds should be returned to full salinity Mediterranean seawater for proper seedling development. For this reason, the seeds that did not germinate were removed from the aquaria to separate containers to maintain the hypo-saline treatment. The salinity in the aquaria was gradually increased to 36-37 of salinity within twelve days. The seeds kept in the separate containers at salinity of 10, continue to germinate and after 15 additional days most of them had germinated. Similarly to the aquaria, the salinity in the containers was gradually increased to reach full salinity. Five weeks after the beginning of the experiment the number of leaves and roots were counted, and the length and width of each leaf and the length of each root were measured for all the seedlings obtained (Fig. 1B). The leaf surface area of each seedling and total root length (sum of all roots per seedling) was estimated.

As the germination of seeds in the full salinity aquaria was very slow and scarce, it was possible to identify each seedling. Each germinated seed was placed individually in glass Petri dishes inside the same aquaria and labelled. Five weeks after germination, the leaves and the roots of each seedling were counted and measured as in the hypo-saline treatment.

Data analysis: the t-test for independent samples was used to compare the cumulative percentages of germination of seeds collected in September 2008 and February 2009 in the two salinity treatments. The t-test for independent samples was also used to evaluate the difference for the number of leaves, the leaf surface area, the number of roots and the total root length of the seedlings between September 2008 and February 2009. Prior to the t-test analysis, the homogeneity of variances was tested by Levene's test. Because the seedling measurements were done at different intervals of time (the "full salinity" seedlings were measured exactly after 5 weeks of germination) while the "hypo-saline" seedlings were measured after 5 weeks of the initiation of the experiment, the early vegetative development

Fig. 1. – Photographs of *Cymodocea nodosa* seeds after hypo-salinity induction. A: seed showing the dorsal ridge opened, and the elongating cotyledon. B: seedling five weeks after the germination.

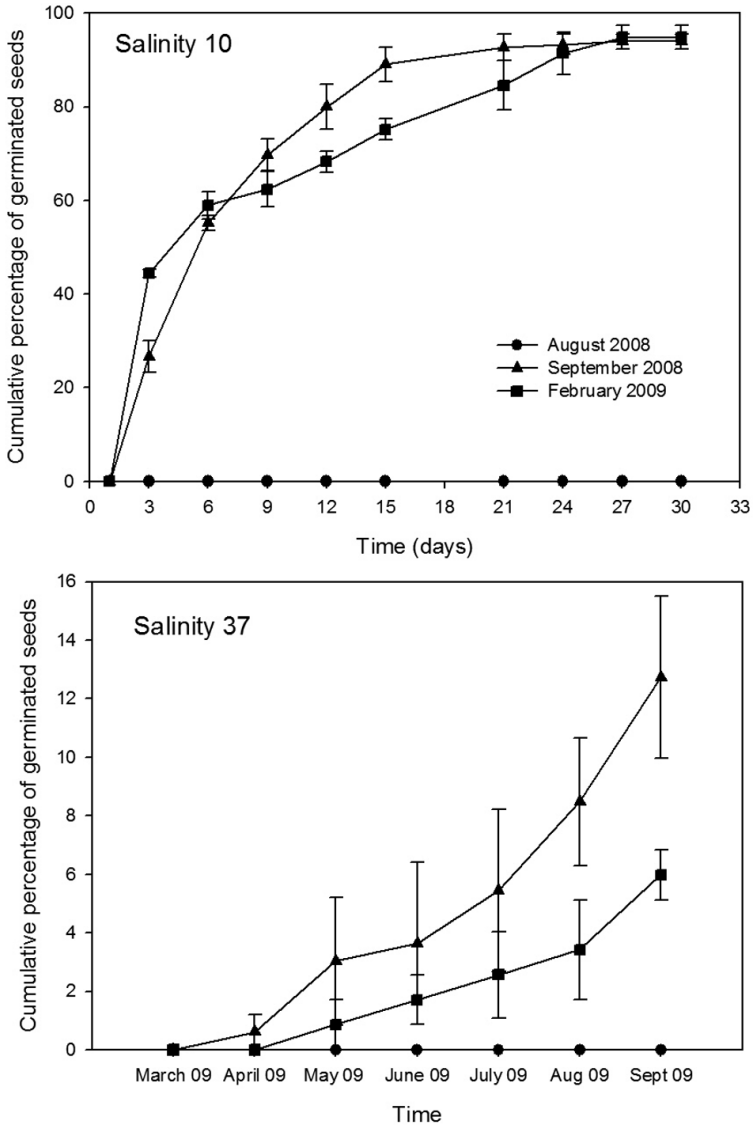

Fig. 2. – Cumulative percentage of germination of *Cymodocea nodosa* fruits/seeds collected in August 2008, September 2008 and February 2009 in the Bay of Pollença (Mallorca, Balearic Islands) after hypo-saline induction of germination (Salinity 10) or kept in full salinity (Salinity 37). The error bars indicate ± 1 standard error of the mean (n = 3). Notice the considerable different scale of Y and X-axes.

achieved by the "hypo-saline" and "full salinity" seedlings was not tested.

RESULTS

Seed germination in hypo-salinity conditions and seedling development

The *Cymodocea nodosa* seeds collected in August 2008 did not germinate and eventually died. On the contrary, the seeds collected in September 2008 and February 2009 started to germinate 3 days after the reduction of salinity, and by the 6th day about half of the seeds in each aquarium had germinated (Fig. 2). The February 2009 seeds germinated faster than the September 2008 seeds showing about 70 % higher percentage of germination by the 3rd day of experiment (t-test: t = -5.11, P = 0.007). The germination rate of the February 2009 seeds slowed down after that and by the 15th day after the initiation of germination, the percentage of germinated seeds was 16 % lower (t-test: t = 3.24, P = 0.032) than that of the September 2008 seeds. No differences of the cumulative percentage of germinated seeds were detected in any other measuring dates (Table I). The maximum percentage of seed germination reached 27 days after the start of the experiment was of 94 ± 1.6 % for the seeds collected in September 2008 and of 95 ± 2.6 % for those collected in February 2009.

Almost all the seeds that germinated by hypo-saline induction developed into a seedling: 99 ± 1.3 % for the September 2008 seeds and 100 % for the February 2009 seeds. The number of leaves, the leaf surface area, the number of roots and the total root length of the September 2008 and February 2009 seedlings were similar after 5 weeks of germination (Table II).

Seed germination and seedling development in full salinity conditions

Similar to the hypo-saline conditions the *Cymodocea nodosa* fruits collected in August 2008 and maintained at salinity 37 did not germinate and died. The seeds collected in September 2008 started to germinate in April 2009 (0.61 %) and those collected in February 2009 started to germinate in May 2009 (0.85 %). During the following months the seeds germinated slowly and by the end of the experiment, in September 2009, the cumulative percentage of seed germination was of 13 ± 2.8 % for the September 2008 seeds and of 6 ± 0.8 % for the February 2009 seeds (Fig. 2). The percentage of germinated seeds tended to be higher in the September 2008 seeds than in the February 2009 seeds but the difference was never significant (Table I).

The percentage of germinated seeds that developed into a seedling was of 61 ± 9.6 % for seeds collected in September 2008 and of 100 % for seeds collected in February 2009 group. The number of leaves, the leaf surface area, the number of roots and the total root length of the seedlings of the September 2008 and February 2009 groups were similar five weeks after germination (Table II).

Table I. – Statistical differences of cumulative percentages recorded in seed germination between dates of collection (September 2008 and February 2009). Significant differences of the t-test results are in bold.

	Times	t	df	P
Salinity 10	Day 3	-5.11	4	**0.01**
	Day 6	-1.14	4	0.32
	Day 9	1.45	4	0.22
	Day 12	2.19	4	0.09
	Day 15	3.24	4	**0.03**
	Day 21	1.35	4	0.25
	Day 24	0.37	4	0.73
	Day 27	-0.31	4	0.77
Salinity 37	April	1.00	4	0.37
	May	0.93	4	0.41
	June	0.66	4	0.54
	July	0.92	4	0.41
	August	1.83	4	0.14
	September	2.32	4	0.08

DISCUSSION

Our results showed that the *Cymodocea nodosa* fruits collected in August 2008 were not mature enough to germinate and produce seedlings. However, in September, one month later, the fruits/seeds were already mature even if most of them were still attached to the shoot. Most of the seeds collected in September 2008 germinated and produced a seedling when the hypo-saline treatment was applied. The seeds collected in February 2009 showed a percentage of germination and an early seedling development similar to that of the seeds collected in September 2008 indicating that they were still mature and no loss of viability occurred after five months. Hence, the collection of mature fruits/seeds of *C. nodosa* in the meadow studied may be performed from September to February. Our results also show that specific care is not needed to maintain *C. nodosa* seeds viable in aquaria. They can be easily maintained in good condition in full salinity seawater and ambient temperature for several months. Consequently it is possible to accumulate a stock of seeds collected during the period from September to February prior to any restoration labours. After germination, the seedlings can also be easily maintained in aquaria with full salinity seawater, at a temperature of 20-21 ºC and a photoperiod of 14:10 h light:dark for at least 5 weeks before planting. Therefore, it is also possible to avoid the harsh growing conditions associated with winter time and produce seedlings in aquaria with at least 3-4 leaves and 1-2 roots ready to be transplanted to the field when the environmental conditions for plant growth improved (spring or summer).

Our results corroborate previous studies that showed that the germination of *Cymodocea nodosa* seeds is to a large extent inhibited by the osmotic pressure of full salinity seawater and that a reduction of salinity may induce seed germination within a few days (Caye & Meinesz 1986, Caye *et al*. 1992). Although these studies concluded that seed germination will occur only if the external osmotic pressure is reduced, we showed that germination of seeds also occurred at full salinity seawater. Differences between locations in the percentage of seeds that germinate at full salinity have been detected previously (Buia & Mazzella 1991) and interpreted as indicators of genetic variability. Partial inhibition of seed germination by the external osmotic pressure provided by full salinity seawater provides a mechanism for the formation of a seed bank, which may be an advantage for *C. nodosa* populations to overcome years of poor reproductive output and to initiate population recovery after disturbances.

The induction of germination of *Cymodocea nodosa* seeds by a hypo-saline treatment may be used for restoration purposes as it allows maximizing the number of seedlings produced relative to the number of mature seeds collected and obtain the seedlings at a faster rate. Seedlings produced after the hypo-saline induction of germination showed a slightly lower vegetative development compared to that of the seedlings produced at constant, full salinity. However, the combined results of the seed

Table II**. –** Vegetative development of *Cymodocea nodosa* seedlings originated from seeds collected in September 2008 and February 2009 after 5 weeks (mean ± standard error, n = 3), and t-test results for the differences between dates of collection.

	Variable	September 08		February 09			
		Mean	± SE	Mean	± SE	t	*P*
Salinity 10	Number of leaves	3.2	0.1	3.1	0.1	0.62	0.57
	Leaf area (cm^2)	1.2	0.2	1.2	0.03	0.58	0.59
	Number of roots	1.6	0.2	1.6	0.1	-0.25	0.81
	Total root length (cm)	1.1	0.2	1.6	0.4	-1.28	0.27
Salinity 37	Number of leaves	4.5	0.05	4.2	0.2	1.21	0.29
	Leaf area (cm^2)	2.0	0.3	2.0	0.4	0.66	0.54
	Number of roots	1.8	0.4	2.2	0.2	0.38	0.72
	Total root length (cm)	1.8	0.9	3.8	0.4	-0.44	0.68

germination and the seedling development make it a good option and interesting alternative for *C. nodosa* restoration and research.

The use of early life stages in restoration is currently an important focus of interest in many parts of the world where seagrass populations have been damaged (Kirkman 1999, Reed *et al*. 1998, Balestri *et al*. 1998, Balestri & Bertini 2003, Holbrook *et al*. 2002, Bull *et al*. 2004, Bos & van Katwijk 2007) and, recently, studies with *in vitro* germinated seedlings (Zarranz *et al*. 2010) regarding to optimizing restoration of *Cymodocea nodosa* have been carried out. Each seedling is a physiologically independent small size unit that may be easily manipulated in field and laboratory experiments, contrasting to large size, clonal adult plants. For restoration purposes the use of laboratory-produced seedlings avoids damage of donor meadows and the small size of the seedlings facilitates their manipulation and planting. In addition, they guarantee the maintenance of a high level of genetic diversity in the restored area (Ruggiero *et al*. 2005).

In summary, the fruits/seeds of *Cymodocea nodosa* in the meadow studied in the Bay of Pollença (Mallorca, Balearic Islands) reach maturity in September even if they are still attached to the parental shoot and can be easily maintained in aquaria at full salinity, a temperature of 20-21ºC, and a photoperiod of 14 h:10 h light:dark for several months. Seed germination by hypo-saline induction (salinity of 10 during 2-4 weeks) reached germination percentages higher than 90 % and the seedlings produced developed 3-4 leaves and 1-2 roots within five weeks when growing in full salinity (37) seawater.

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