

Timing and success of reproductive stages in the seagrass *Zostera noltii*

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Abstract

The timing and success of sexual reproduction of the seagrass *Zostera noltii* was investigated at the Ria Formosa lagoon, Portugal. Thirty plants were tagged and monitored individually through time to determine *in situ* the development time of each maturation stage, from the emergence of the flowers to the production of seeds. The overall process of flowering and fruiting lasted 47 ± 4 days, during which formation and maturation of the fruits was the most time-consuming stage (27 ± 2 days). Spathe success, i.e. the percentage of spathes that produced seeds, was $22 \pm 4\%$ while spathe mortality was $34 \pm 6\%$. A considerable percentage of spathes ($37 \pm 7\%$) was lost through leaf detachment, but some of these may still add to the reproductive success of the species by contributing to the species recruitment within the source meadow or elsewhere. Meadow seed production (MSP) of *Z. noltii* was estimated to be 312 ± 66 seeds m^{-2} , whereas the potential seed production of the studied meadow was 2623 seeds m^{-2} . Under laboratory conditions, 70% of *Z. noltii* seeds germinated within 26 days, but only 10% reached the seedling stage due to the high mortality of germlings. Fertility, defined as the probability of a seed to originate a new plant, was estimated to be 14×10^{-4} , which is higher than what is expected for most seagrasses.

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1. Introduction

Seagrasses are capable of reproducing both sexually and asexually. Vegetative development through clonal growth has been reported to be the main process for maintenance and establishment of seagrass meadows (Rasheed, 1999; Hemminga and Duarte, 2000; Plus et al., 2003; Rasheed, 2004). Flowering events and seed survival have been considered to be important mechanisms to maintain genetic diversity, possibly being the only way to colonize bare sediments or disturbed areas (Marbà and Walker, 1999; Orth et al., 2000; Plus et al., 2003). However, recent developments in genetic tools that allow the distinction of ramets and genets have revealed the occurrence of meadows where sexual recruitment contributes more than vegetative propagation for meadow composition. The balance between both types of reproduction varies from meadow to meadow even within the same species, ranging from monoclonal (no sexual contribution) to populations where every sampled shoot came from a different seed (e.g. Coyer

et al., 2004; Olsen et al., 2004; Arnaud-Haond et al., 2005). The reasons for such large variation in the relative importance of sexual reproduction between populations are not known, but may be related to contrasting levels of success of the various stages comprised in the production of sexual recruits.

Successful recruitment from sexual reproduction is a rare event in several seagrass meadows. The existence of bottlenecks along the reproductive cycle of seagrasses has been suggested to constrain their reproductive success, particularly in the main steps such as flowering, fruiting, seed production, germination and seedling development (Hemminga and Duarte, 2000). The reproductive biology of *Zostera noltii* has been reported for Mediterranean populations (Buia et al., 1985; Loques et al., 1988; Loques et al., 1990; Buia and Mazzella, 1991; Goubin and Loques, 1991; Curiel et al., 1996) and for southern Portugal (Alexandre et al., 2005). Despite the reproductive structures and the sequence of the steps involved in the process being well described, the success of seed formation and the timing of all stages involved from the emergence of flowers to the production of seeds have not been previously studied, although they may represent a bottleneck in the *Z. noltii* reproductive cycle. The conservation and restoration of seagrass meadows using seeds has proven to be a

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challenging task requiring a thorough understanding of the timing and bottlenecks in the different stages involved in seed formation.

This study aimed to characterize the timing and success of the stages of sexual reproduction of the seagrass *Z. noltii* at the Ria Formosa coastal lagoon, southern Portugal. In particular, the following reproductive characteristics were estimated: (i) the time elapsed between maturation stages of the inflorescences in the field; (ii) the success, mortality rate and loss of the spathes; (iii) spathe production, seed production and potential seed production; and (iv) seed germination and survival rates of germlings.

2. Methods

2.1. Study site

This study was conducted during summer 2003 in the Ria Formosa lagoon, southern Portugal (37°01'N, 7°51'W), in an intertidal meadow of *Z. noltii*. This meadow is under the direct influence of the nearby inlet of Armona. Ria Formosa is a mesotidal coastal lagoon covering an area of about 84 km². The tidal amplitude ranges from 3.50 m on spring tides to 1.30 m on neap tides (Andrade, 1990). Water temperature in the lagoon varies between 12 °C during winter and 27 °C in the summer. Salinity ranges from 35.5 to 36 PSU along the year except during heavy rainfalls, when can be as low as 15 PSU (Falcão, 1996). The seagrass *Z. noltii* is widely dispersed along the intertidal areas of the lagoon.

2.2. Tagging method

In order to determine the time elapsed from the emergence of flowers to the production of seeds, as well as the time elapsed within intermediate steps of the cycle, a tagging method was applied to each flowering shoot individually. Flowering shoots

Table 1
Maturation scale of the flowers inside the spathes (adapted from Alexandre et al., 2005)

	Maturation stage	Main characteristics
Flowering	0	Undefined flowers inside the immature inflorescence
	I	Flowers aligned on a single stem; sheath closed
	II	Mature females erected outside sheath for fertilization
	III	Mature males release filiform pollen (anther dehiscing)
	IV	Fertilized females, thecae empty (if still present)
Fruiting	V	Presence of fruits
	Va	Small embryos inside the female flowers
	Vb	Fully developed fruit
	Vc	White fruit presents a thin coat
	Vd	Greenish-blue fruit with a hard brownish-black coat: seed stage

were randomly tagged ($n = 30$), during low tide, with plastic rings around the shoot peduncle. The plastic rings were placed around the shoot base at the sediment surface, to avoid rhizome damage. Flowering shoots were monitored in the field every week throughout the fertile season, from early June to late July 2003. Spathes of each flowering shoot were observed and their maturation stage was recorded to estimate the time elapsed between the maturation stages. Spathe maturity was classified based on the maturation scale developed by Alexandre et al. (2005). During the field observations we found a substantial number of spathes in a more immature stage than stage I in the maturation scale of Alexandre et al. (2005). Flowers inside the spathes had no articulate morphology yet, but rather looked like a small, white, mass of tissue. Consequently, a new maturation stage (stage 0), was considered to account for these immature spathes (Table 1). When most of the spathes within a flowering shoot were in an advanced stage of maturity (stage V), the shoot was carefully removed and transported to the laboratory. Flowering shoots were removed at this point to prevent natural shoot detachment and seed loss, which happens when the shoots reach the seed stage. The flowering shoots were placed separately inside plastic containers and were kept in an aerated natural seawater aquarium within a culture chamber, at field temperature (23 °C) and salinity (35 PSU) conditions. The maturation stages of the spathes of each flowering shoot were monitored weekly, until late August.

2.3. Characterization of reproductive success

Reproductive success of the flowering shoots was estimated as the percentage of flowering shoots that produced seeds, i.e. the percentage of shoots that attained stage Vd (Table 1). Spathe success was defined as the percentage of spathes that produced seeds, i.e. spathes that reached stage Vd. Spathe mortality was estimated as the percentage of spathes that showed signs of necrosis before forming seeds. Spathe loss was estimated as the percentage of spathes that was tagged but not found in subsequent visits. Spathe production was estimated as the number of new spathes produced per flowering shoot per day during the overall monitoring period. Seed production was calculated as the number of seeds produced per flowering shoot. Meadow seed production (MSP) was estimated as:

$$\text{MSP} = \text{SP} \times \text{FSD}$$

where SP is the seed production per flowering shoot and FSD is the density of flowering shoots of *Z. noltii* at Ria Formosa, obtained from previous work on the same meadow and during the same period (126.3 ± 43.2 flowering shoots m⁻²; Alexandre et al., 2005).

2.4. Seed germination and germling survival

To evaluate the germination rate of *Z. noltii* seeds, we used seeds that had fallen off naturally from the spathes ($n = 41$). Seeds were incubated individually in assay tubes and the source flowering shoot was noted. This step allowed us to follow the seeds individually and to investigate whether seed viability was

Table 2
Time elapsed (days) between maturation stages in *Zostera noltii* spathes

Maturation stages	Mean \pm S.E.	Range
0–Vd	47.3 \pm 3.8	40–57
I–Vd	40.4 \pm 3.2	28–50
I–IV	10.0 \pm 0.7	6–22
Va–Vd	27.0 \pm 2.1	17–43

independent of the parental flowering shoot. Seeds were maintained under optimal germination conditions (1 PSU and 22 °C) according to Hootsmans et al. (1987) and Loques et al. (1990), and monitored daily during the first 10 days and every 2 days until the end of the experiment. We defined germination as the spontaneous rupture of the seed coat and emergence of the cotyledon (Hootsmans et al., 1987). After germination, the germlings were kept at a salinity of 10 PSU and at the same temperature (Loques et al., 1990) until they reach the seedling stage, i.e. when the first chlorophyll-bearing leaves appeared. At that stage, the seedlings were changed to a salinity of 20 PSU. After 76 days of experiment, the seeds that did not germinate were tested for viability. The seeds were soaked in a 0.5% tetrazolium solution at 25 °C during 24 h (Conacher et al., 1994). Seeds were considered viable if the hypocotyl stained red and the radicle either red or brown.

3. Results

3.1. Time elapsed between maturation stages

The time elapsed between maturation stages is shown in Table 2. The overall process of flowering and fruiting (stages 0–Vd), from the inflorescence emergence to the seed formation, was completed within 47.3 ± 3.8 days. The time required for fruit development and maturation (stages Va–Vd) was longer (27.0 ± 2.1 days) than that required for the pollination process (stages I–IV), which was completed within 10.0 ± 0.7 days.

3.2. Characterization of reproductive success

On average, the reproductive success of the flowering shoots was 63.3% (Table 3). Spathe success was $22.4 \pm 4.1\%$, while

Table 3
Reproductive success of flowering shoots and spathes, spathe mortality, loss and production, seed production, and potential seed production for *Zostera noltii* in the Ria Formosa ($n = 30$, except for seed production ($n = 17$))

	Mean \pm S.E.	Range
Flowering shoot success (%)	63.3	
Spathe success (%)	22.4 ± 4.1	0–75
Spathe mortality (%)	34.2 ± 6.4	0–100
Spathe lost (%)	36.6 ± 6.9	0–100
Spathe production (# spathes day ⁻¹)	0.023 ± 0.005	0.00–0.09
Number of spathes (# spathes flowering shoot ⁻¹)	4.20 ± 0.24	2–8
Seed production (# seeds flowering shoot ⁻¹)	2.47 ± 0.52	0–7
Meadow seed production (# seeds m ⁻²)	312.1 ± 66.0	0–884.3

spathe mortality was 34.2%. A considerable percentage of spathes were lost through leaf detachment, $36.6 \pm 6.4\%$, but some of these may still add to the reproductive success of the species, contributing to the species recruitment within the source meadow or elsewhere.

The average number of spathes produced per flowering shoot was 0.023 ± 0.005 day⁻¹. Each flowering shoot produced an average of 2.47 ± 0.52 seeds (Table 3), whereas the meadow seed production in *Z. noltii* was 312.1 ± 66.0 seeds m⁻² (Table 3).

3.3. Seed germination and germling survival

Germination only occurred until the 26th day of the experiment (Fig. 1A). During this period, several germination peaks were observed, reaching maximal values of 12% at the first and ninth day of the experiment. On the whole, 70% of the seeds germinated. The seeds that did not germinate were not viable as they did not stain with tetrazolium. Of all germinated seeds, only three (10%) reached the seedling stage. The time required by a germinated seed to become a seedling varied from 17 to 23 days, whereas the life-time of a seedling varied from 21 to 50 days. The individual that survived longer did so for 72

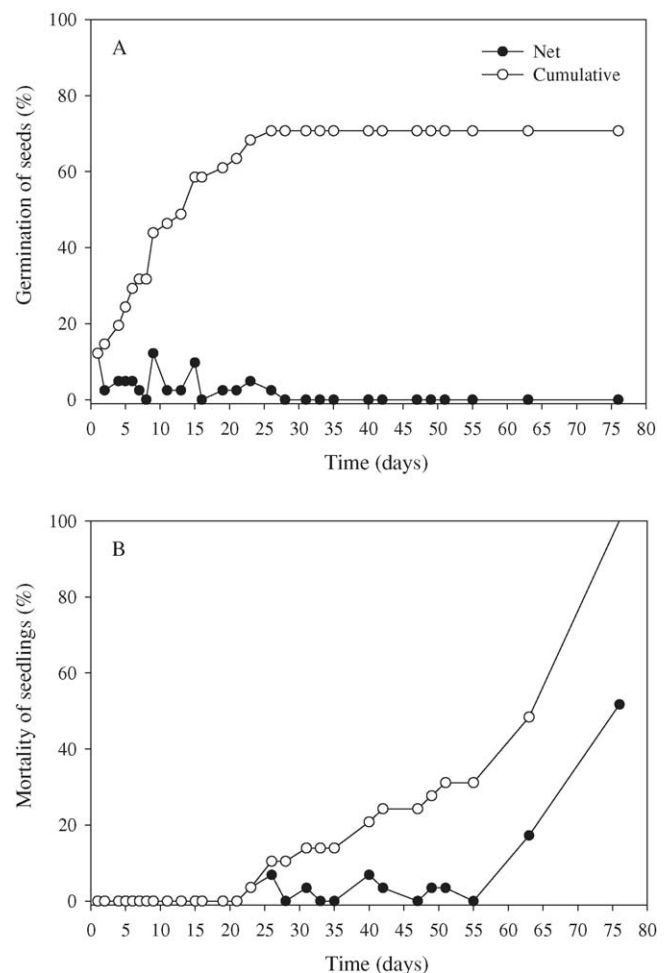


Fig. 1. Net and cumulative germination of *Zostera noltii* seeds at 1 PSU (A), and mortality of seedlings (B).

days. Mortality of germlings and seedlings (Fig. 1B) occurred between the 23rd and the 76th day of the experiment. Initially, only small mortality peaks were observed, until the greatest mortality events that occurred on the 63rd day (17%) and on the 76th day (52%), when all the germlings and seedlings died and the experiment ended.

Seed viability was independent of the parental flowering shoot, i.e. germination percentages of seeds were not significantly different among parental flowering shoots ($\chi^2 = 8.46$, $P > 0.05$), thus providing no evidence for parental effects.

4. Discussion

Data obtained on the flowering and fruiting of *Z. noltii* showed that the flowering process (stages I–IV) is the shortest step, which is completed within 10 days. Stage III, which is related to pollen release, was rarely observed because it is an ephemeral event. This process may occur within a few hours, as observed by De Cock (1980) for *Z. marina*. The formation and maturation of the fruits (stages Va–Vd) is the most time-consuming step (27 days), which was also similar to that reported for *Z. marina* (26 and 28–30 days by De Cock (1980) and Silberhorn et al. (1983), respectively). This step was expected to take longer than the first maturation stages because it comprises the processes from the embryo to seed formation. This last stage includes the accumulation of resources received from the mother plant necessary for future seedling development (Baskin and Baskin, 1998; Fenner and Thompson, 2004). The time required for the overall process of flowering and fruiting of *Z. noltii* (47 days) is slightly longer than that observed for *Z. marina* (30–40 days; De Cock, 1980). These time differences may be related to the life strategy of the species/populations. The populations of *Z. marina* studied by De Cock (1980) present an annual life cycle, whereas *Z. noltii* in the Ria Formosa is perennial. The flowering season of *Z. marina* from higher latitudes is shorter (late July to October, Hootsmans et al., 1987; March to June, Olesen, 1999) than that of *Z. noltii* from the Ria Formosa (March to November, Alexandre et al., 2005), which may explain the faster reproductive process observed in *Z. marina*.

Sixty three percent of the flowering shoots of *Z. noltii* produced at least one seed. However, only about one third of the spathes was successful, i.e. produced at least one seed. Spathe mortality was relatively high (34%), representing a considerable plant investment into seed production that was lost, but spathe loss (37%) may still contribute to the species sexual recruitment within the meadow or elsewhere, depending on the fate of the detached flowering shoots that carry spathes. Fruit maturation and seed formation may proceed in detached spathes, or spathes may just degenerate and die. No information is available on this subject.

The meadow seed production obtained in this study (312 ± 66 seeds m^{-2}) is much lower than the potential seed production estimated for the studied meadow (2623 seeds m^{-2}), i.e. the average number of seeds produced per flowering shoot multiplied by the maximum flowering shoot density (1062

flowering shoots m^{-2} ; Alexandre et al., 2005). This value was twice than that estimated by Curiel et al. (1996) for the same species at the lagoon of Venice (1300 seeds m^{-2}), but much lower than the potential seed production estimated at the Netherlands (9000 seeds m^{-2} , Hootsmans et al., 1987). This high reproductive potential does not guarantee high success of the reproductive cycle, i.e. the formation of new plants. Germination of seeds is a complex process regulated by environmental factors such as salinity and temperature (Hootsmans et al., 1987; Loques et al., 1990) or by physiological factors such as dormancy processes or lack of viability (Conacher et al., 1994; Orth et al., 2000). Beside these factors, seed loss may be caused by physical damages, exports to unsuitable areas, burial and predation (Keedy and Patriquin, 1978; Harrison, 1993; Fishman and Orth, 1996; Peterken and Conacher, 1997). Under laboratory conditions, 70% of *Z. noltii* seeds germinated within 26 days, which was higher and faster than the germination found by Loques et al. (1990) for the same species (42% within 63 days). Remaining seeds did not germinate due to the lack of viability. The high rate of seed germination observed here under laboratory conditions does not necessarily reflect natural values in the field. However, low salinity conditions that trigger *Z. noltii* seed germination can occur in the intertidal zone sporadically during low tide, after heavy rainfall, suggesting that seed germination may occasionally be very high.

Among all the germinated seeds, only about 10% reached the seedling stage due to the high mortality of germlings. Considering that the germination rate of the seeds was 70%, we estimate that the reproductive success of *Z. noltii* in Ria Formosa to the seedling stage can be up to 7%. However, the reproductive success to a new adult plant (fertility) will be much lower. Many seagrass populations have been reported to have a seedling survival of less than 2% (Duarte and Sand-Jensen, 1990; Harrison, 1993; Olesen and Sand-Jensen, 1994; Olesen, 1999). This would result in fertility values for *Z. noltii* of the order of 14×10^{-4} , which is higher than what is expected for most seagrasses, well below 10^{-5} (Hemminga and Duarte, 2000). In the Netherlands, fertility values in *Z. noltii* were estimated to be 5% (Hootsmans et al., 1987). Furthermore, recent genetic studies have reported that sexual reproduction can be a major recruitment process in *Z. noltii* (Coyer et al., 2004; Diekmann et al., 2005).

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