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Short communication

Long-term seed storage and viability of *Zostera marina*

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ABSTRACT

Successful establishment of seedlings in populations of *Zostera marina* (eelgrass), especially for restoration efforts using stored seeds, depends in part on viability and germination of seeds. Seeds of *Z. marina* were collected from plants and stored in seawater at 5 °C for up to several years. Seed viability, assessed with the viability stain, tetrazolium chloride, decreased steadily over a four-year period. There was a strong correlation between age and viability; viability of fresh seeds was approximately 77% whereas for four-year old seeds was 32%. However, only 51 of 975 of fresh seeds that germinated (~5%) developed leaves. The physical structure of the seed was evaluated to understand the effects of aging. It was determined that as seeds age there is an increase in the number of fractures on the seed coat. These data combined with the recent awareness that global seagrass populations are declining present valuable information to help maintain viable seed repositories which may contribute to the conservation and restoration of these wild plants.

1. Introduction

Appropriate seed storage methods were, and still are, critical to providing an adequate supply of food for human beings and domestic animals (Biasutti Owen, 1956; Hong and Ellis, 1996; Nabhan, 2009). Both short term storage from harvest to planting in the following year, and long term seed preservation are necessary to insure crop sustainability (Hong and Ellis, 1996). Those engaged in the restoration of important wild plant species also recognize the value of developing seed storage techniques (Young and Young, 1986; Guerrant et al., 2004). While there are general conditions such as temperature and moisture that must be considered to ensure seeds do not degrade, the response of seeds to a storage environment can vary widely among species (Hong and Ellis, 1996; Young and Young, 1986; Guerrant et al., 2004). The response of seeds to storage conditions must be evaluated on a species by species basis before a specific storage technique is recommended. Knowledge related to seed storage for wild plants becomes more important with the advance of seeding programs to restore the valuable habitat they provide (Guerrant et al., 2004).

The restoration of seagrasses or marine flowering plants into areas globally threatened by human and other activities (Short and Wyllie-Echeverria, 1996; Orth et al., 2006a; Waycott et al., 2009) now benefits from programs that deliver seed to the seafloor (reviewed in Orth et al., 2006b, 2012). Moreover, the collection, processing, short-term storage and sowing of seeds from a single species is now an accepted restoration practice (Pickerell et al., 2005; Marion and Orth, 2010; Busch et al., 2010; Tanner and Parham, 2010). However while these techniques have clarified issues related to short-term storage (<1 year) little is known about the viability of seeds over longer periods of time.

In earlier work, McMillan (1991) found, by measuring percent germination in the laboratory, that some portion of the seeds of *Halodule wrightii*, *Halodule univervis*, *Syringodium filiforme* and *Halophila engelmannii* were viable for between two and four years depending on the species. Additionally, Zipperle et al. (2009) found that *Zostera noltii* seeds persist in the wild up to three years, however this work has not been replicated and no other studies discuss the behavior of *Zostera marina* seeds in long-term storage treatments. This information may be a critical component of future restoration efforts (e.g. Walmsley and Davy, 1997; Liu and Spira, 2001).

The primary goal of our research was to determine the feasibility of long-term seed storage for *Z. marina*. Objectives were first to determine seed viability, using both germination and viability staining, for seeds stored over several years, and second to evaluate seed coat behavior in long-term storage using SEM.

2. Materials and methods

2.1. Seed collection

Z. marina generative shoots were collected in the Fall of 2005 through 2010 from False Bay, San Juan Island, Washington State,

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USA. Generative shoots were placed in outdoor tanks serviced by flowing seawater at Friday Harbor Laboratories (FHL), University of Washington. Once released, seeds were collected by sieving tank water. Collected seeds were placed in 20 ml seawater in scintillation vials, in batches of 100, and stored in the dark at 5 °C until experiments were initiated. Seeds for experiments were randomly selected from stock and placed into treatments.

2.2. Seed metrics

Each seed used in experiments described, plus 168 seeds haphazardly selected each year (2005–2011) from fresh seeds collected in the field were measured to determine seed size classes (Wyllie-Echeverria et al., 2003). To do this the length and width of each seed was measured to the nearest 0.05 cm. These measurements allowed us to determine seed weight using the model:

$$Y = ax + b$$

where Y = seed weight (mg), $a = 2.01$, x = cross-sectional area in mm^2 and $b = -2.4$ (Wyllie-Echeverria et al., 2003).

After seed weights were determined individual seeds were assigned to a small, medium or large size class (Wyllie-Echeverria et al., 2003).

2.3. Determination of seed viability by staining

Seed viability was assessed by immersing seeds in a 1% (w:v) tetrazolium chloride (TTC) solution. The TTC test is an internationally accepted test to determine seed viability and has been previously used with success in seagrasses (e.g. Conacher et al., 1994; Alexandre et al., 2006; Cabaço and Santos, 2010). Using a sterile scalpel, a small incision was made in the seed coat to allow the TTC to enter. After absorbing TTC the embryo, if viable, undergoes a redox reaction changing color from white to reddish brown (Supplemental 1) during cellular respiration (Smith, 1951). When we initiated the study in 2008 fifty seeds from each year (2005–2008) were tested. This test was repeated in 2009, 2010 and 2011. Before immersion, length and width (to the nearest 0.05 mm) and weight (to the nearest 0.05 mg) of each seed were recorded (Wyllie-Echeverria et al., 2003). During TTC treatment, seeds were placed in the dark and held at 20 °C (Phillips, 1972). Twenty-four and 48 h later, the number of viable seeds (based on embryo color (Supplemental 1)) in each batch was recorded.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquabot.2013.06.006.

2.4. Determination of seed viability by germination

On 14 February 2009 germination experiments were initiated with two replicate treatments ($n = 35$ seeds in each treatment) for each year (2005–2008). Each seed was placed in a petri dish filled with 25 ml, sterile seawater (10 PSU). Petri dishes were randomly distributed on a rack and kept in the dark in a climate-controlled room [10 °C, 1 ATM, ~50% relative humidity]. The development of each seed to seedling was monitored between 14 February and 1 June 2009. In addition, 975 fresh seeds were placed in uncapped, plastic test tubes serviced by fresh nutrient enriched seawater medium [$NaNO_3 + Na_2HPO_4 + MnCl_2{\cdot}4H_2O$ + ferric-sodium EDTA + H_3BO_3 + HCl] (AC Churchill unpublished data) held at 15 °C. Seeds were allowed to germinate and grow to determine the relationship between seed viability and seedling development. For the purposes of this experiment, we defined germination as the stage when hypocotyl extension occurs (see Churchill, 1992, and Supplemental 2). After the seed germinated it was monitored for the development of leaves.

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Table 1
Mean seed size metrics ± SE ($n = 168$ each year).

Year	Length (mm)	Width (mm)	Weight (mg)
2005	3.75 ± 0.18	1.71 ± 0.16	7.76 ± 1.28
2006	3.71 ± 0.18	1.63 ± 0.13	7.14 ± 0.96
2007	3.88 ± 0.23	1.62 ± 0.11	7.52 ± 1.02
2008	3.74 ± 0.15	1.65 ± 0.14	7.31 ± 0.94
2009	3.82 ± 0.19	1.61 ± 0.13	7.34 ± 0.86
2010	3.80 ± 0.18	1.59 ± 0.11	7.13 ± 0.90
2011	3.62 ± 0.23	1.61 ± 0.13	6.84 ± 1.14

2.5. Scanning electron microscope (SEM) images of stored seeds

SEM images were taken of randomly selected seeds representing various years of storage; eleven seeds were scanned for 2011, ten for 2010, 2007 and 2006. Seeds from 2008 and 2009 were not available. The seeds were obtained from the same seed stock used in germination experiments. Each seed was individually prepared, mounted and scanned using the following standard procedure: (1) seeds were gently blotted with a Kimwipe until dry; (2) dry seeds were mounted with conductive tape to a specimen block; (3) seeds were coated with gold/palladium using a Sputter Coater; and (4) each seed was individually scanned using JCM-5000 NeoScope by JELO. Scanned seeds were compared to each other by counting the number of fractures in the seed coat, and by assigning a value using the classification chart (Table 3).

2.6. Statistical analysis

Statistics were computed in R (R version 2.14.2). A chi-squared test was performed to investigate the different means. A GLM model was developed and one-way ANOVA was computed to analyze the seed fractures data and seed class differences.

3. Results

3.1. Seed size classes

Mean seed length, width and weight remained relatively constant from year to year (Table 1). Mean seed weight was more variable, especially when comparing 2005 weights to 2011 weights; however this difference was not statistically different. Except for 2005, seed size classes were also relatively constant (Table 2). In 2005 there were more large-sized seeds than the other two classes combined, which was significantly different from other years ($\chi^2 = 213.29$, df = 12, $P < 0.001$).

Table 2
Seed size frequency per year ($n = 168$ each year).

Year	Small	Medium	Large
2005	5	78	85
2006	6	149	13
2007	4	137	27
2008	4	149	15
2009	3	152	13
2010	4	148	16
2011	18	134	16

Table 3
Seed classification chart concerning the integrity of the seed coat.

1	2	3	4	5
Seed is intact, few to no fractures, ridges are continuous and sealed, germination is highly likely	Seed is mostly intact, few fractures, ridges are continuous and sealed, germination is likely	Seed is partially intact, few fractures, ridges are mostly continuous, germination is somewhat likely	Seed is highly fractured and not entirely intact, ridges are semi-continuous and germination is unlikely	Seed is not intact, multiple fractures, ridges are no longer continuous and germination is highly unlikely

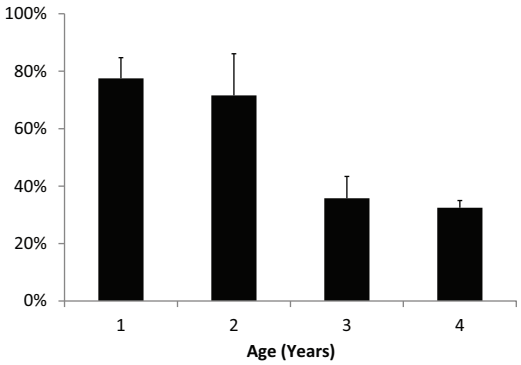

Fig. 1. Seed viabilty by year, determined by TTC-test (mean ± SD).

3.2. Determination of seed viability by staining

Combined mean seed viability for 2005–2008 was 54.3% but with a large variation between years. Maximum viability was 77% for the year-old seeds. The viability of two-year-old seeds only dipped slightly to 71%. However after three years the viability decreased to 37%, and dropped to 31% in the fourth year. There was a strong and significant relationship between age and decrease in viability (Fig. 1; $\chi^2 = 23.03$; $P < 0.001$). There was a positive correlation between seed size and viability. Medium and large seeds have a higher percent viability than the smaller seeds. This relationship holds true except in the case of year four when the large seeds may not, in fact, be large seeds but smaller ones that have bloated due to breakage in the seed coat (SEM images support this conjecture).

3.3. Determination of seed viability by germination

Germination rates of *Z. marina* were consistent in trend but lower than viability rates. One-year-old seeds had the highest germination rate at 68% and dropped to 15% after three years ($\chi^2 = 22.04$, $P < 0.001$) (Fig. 2). Hypocotyl extension followed the same trend, decreasing with each consecutive year ($\chi^2 = 25.3$, $P < 0.001$). These data demonstrate that although a seed may be viable, it still may not produce a seedling. There was a high frequency of embryonic abortion; of the 975 fresh seeds used in this study only 51, i.e. ~5% produced successful seedlings growing more than 10 cm. In older seeds, germination was often followed by fungal infection and embryonic failure.

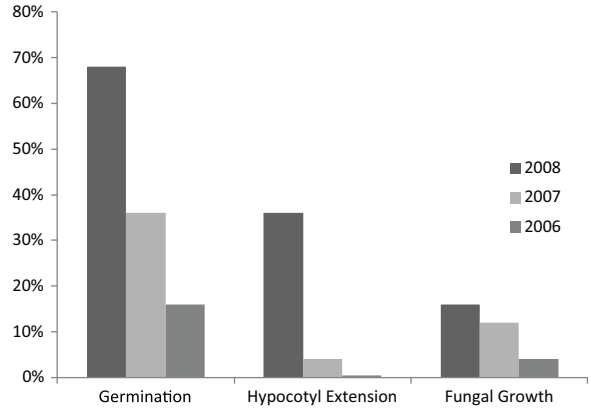

Fig. 2. Data show percent of sample showing each trait: germination, hypocotyl extension and fungal development over time.

Table 4
Summary of seed classification, using Table 3 and number of fractures observed in seed coat (mean ± SD).

Year	Classification	Number of fractures
2011	1.5 ± 0.7	1.8 ± 2.1
2010	1.3 ± 0.4	0.4 ± 0.9
2007	3.7 ± 1.2	2.6 ± 1.5
2006	3.2 ± 1.0	3.3 ± 2.8

3.4. Scanning electron microscope (SEM) images of stored seeds

We determined that the *Z. marina* seed coats are formed from an epidermal layer of cells (Moïse et al., 2005) that forms multiple ridges and valleys around the seed (Fig. 3a). It appears as if seed-coat separation from the embryo during germination occurs along the ridges (Fig. 3a) (Anderson et al., pers. comm.). For the purposes of this study we examined the seed coat as a proxy for permeability of the seed coat structure over time (Table 3). Results are listed in Table 4. It was determined that young seeds had few if any fractures in the seed coat (Table 4; Fig. 3b). Fractures, when observed, occurred perpendicular to the ridges and occurred with more frequency as the seed aged ($P = 0.009$; Table 4: Fig. 3c–g). Additionally, using the classification chart the difference between years, listed in Table 2, was highly significant ($P < 0.001$).

4. Discussion

Although seagrasses take advantage of asexual growth to maintain extant meadows (Tomlinson, 1974), the seed rain carries propagules to suitable habitat adjacent to existing populations or more distant locations (Orth et al., 2006b). This reproductive strategy can contribute favorably to restoration programs. For example the collection, processing, short-term storage and sowing of *Z. marina* seed is now an accepted and successful restoration practice (Pickerell et al., 2005; Marion and Orth, 2010; Busch et al., 2010; Tanner and Parham, 2010). Techniques associated with this effort are now being used to develop protocols to restore other seagrass species using seeds (Dominquez et al., 2010; Zarranz et al., 2010; Kishima et al., 2011). However, while this research describes procedures to store seeds effectively from collection to planting in the same year, except for early work by McMillan (1991), no information related to the behavior (expressed as percent viable) of seeds stored for a longer time period is available. Research related to seed behavior in long-term seed storage for terrestrial flowering plants describes the need to develop procedures to ensure long-term storage does not degrade seed condition (Hong and Ellis, 1996; Guerrant et al., 2004). Our work demonstrates that while *Z. marina*

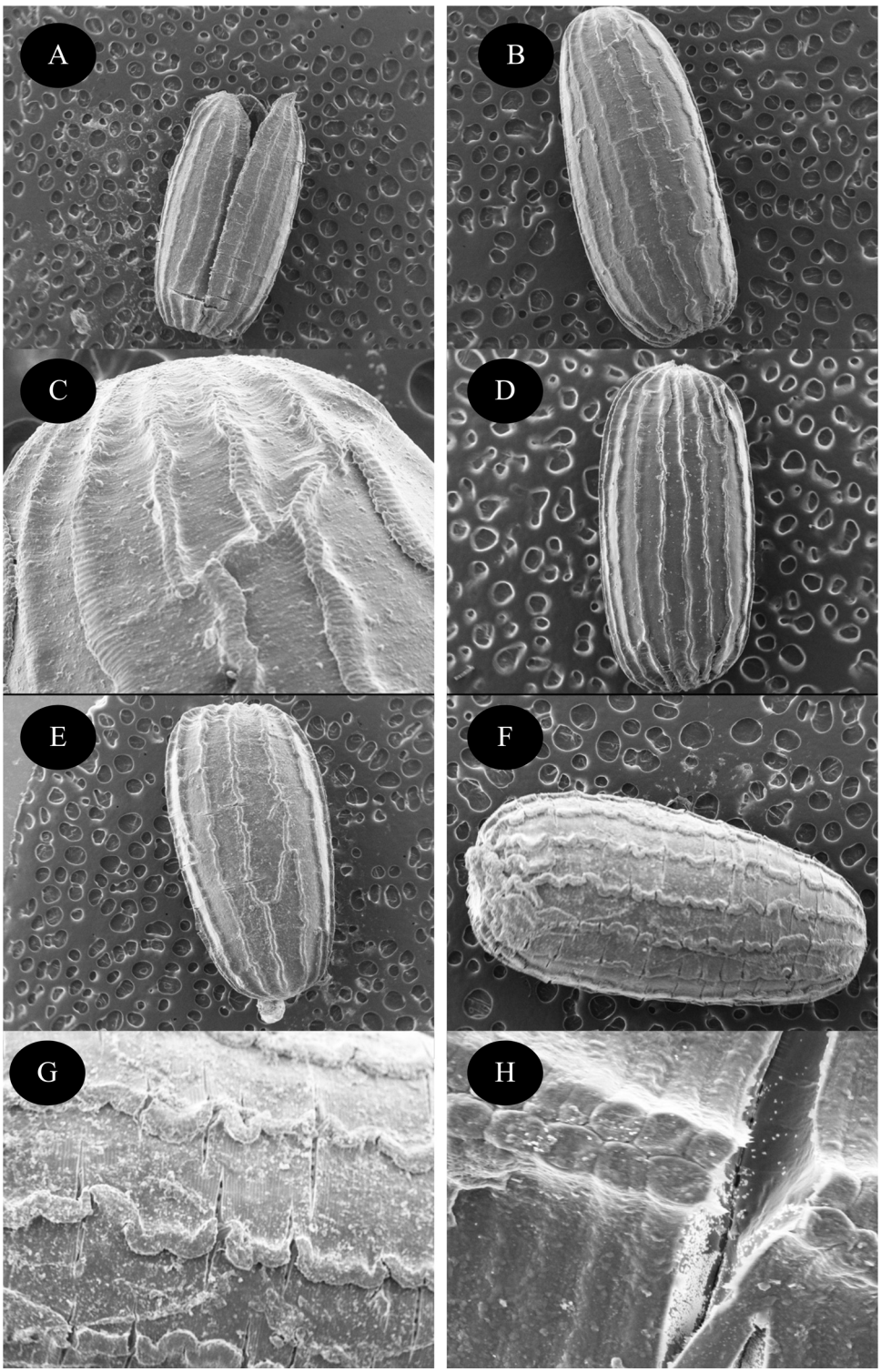

Fig. 3. (a) Separation of the seed coat from the embryo. Notice the separation occurs along the ridge. (b) and (c) SEM image of a fresh seed (1 year old). Notice that the seed surface is made up of ridges and valleys. There are no observable cracks in the surface of the seed. (d) Two year-old seed. Notice that there are still few fractures within the seed coat however at the top of the seed the seed coat is opening. (e) Three year-old seed. Notice there are more fractures within the seed coat; and the embryo extends out through an opening at the end of the seed. (f) and (g) Four year-old seed. Notice that there are an abundant amount of fractures within the surface of the seed. The ridges are no longer continuous nor are they straight along the seed length. (h) SEM image of *Z. marina* seed at 1000×. This is a close up of a ridge along the seed with a fracture going through the ridge.

seeds appear to be viable during storage for at least three years, as observed for the natural seed-banks of *Z. noltii* (Zipperle et al., 2009), only 15% of these germinated. Further, viability is reduced after two years and only a fraction of those seeds will produce a seedling.

In this experiment we demonstrate that if each flowering shoot produces approximately 20 viable seeds (Wyllie-Echeverria et al., 2003), and only 5–10% of viable seeds produce a seedling (Harrison, 1993; Cabaço and Santos, 2010) then one-year old seeds will only produce ~1 seedling per flowering shoot [equation = 22(seeds per

shoot) × 0.78(viability) × 0.075(average viability to seedling ratio)], and by year three it will require multiple flowering shoots to produce one new seedling. We postulate that this viability reduction is linked to several different factors, and represent an important bottleneck in the species life-strategy. For example, it is known that seeds require a minimum amount of energy and hormones present to complete germination (Finch-Savage et al., 2006); however, if hormones or stored lipids, sugars and starches, are degraded or leached through fractures in the seed coat, as observed in SEM images, viability of older seeds could be compromised. In a pilot experiment we found that exposing older seeds to gibberellin (10^{-9} M) increased ($P < 0.001$) the frequency of germination. SEM evaluation of seed coat integrity suggests that fractures in the coat as the seed ages result in embryonic death. While we lack information to describe the biochemical process forcing seed coat fracturing, one obvious line of inquiry would be to explore the relationship between various storage media and environments on seed viability over time.

The increase in projects to restore *Z. marina* using seeds was guided by a series of publications to assist practitioners with collection, short-term storage and sowing techniques (Pickerell et al., 2005; Orth et al., 2006c). These publications form the basis for an *operations manual* to continue *Z. marina* restoration projects and potentially direct program development for other seagrass species. Consequently we recommend the same process be considered here. That is, it seems wise to compile an operations manual that explains a step-wise procedure to prevent the degradation of seagrass seeds in long-term storage. Our recommendation is based in the awareness that the global seagrass decline is on the rise (Short and Wyllie-Echeverria, 1996; Orth et al., 2006a; Waycott et al., 2009) and viable seed repositories contribute to the conservation and restoration of wild plants (Nabhan, 1989).

Conflict of interest

The authors declare no competing financial interest.

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