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Short communication

The effect of storage condition on viability of *Enhalus acoroides* seedlings

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ABSTRACT

Using nursery stock grown from seeds rather than transplants could improve success of seagrass restoration. In Indonesia, *Enhalus acoroides* is the climax seagrass species and the target species for restoration. However, optimal conditions for seed storage are not known. The aim of this study was to determine optimal storage conditions for *E. acoroides* seeds to maximize germination and growth. Seed germination was examined under different lengths of storage and storage temperatures (room and refrigerator) for *Enhalus* fruits (with the seed inside). *Enhalus* seeds did not survive well and turned black after 2 days in the refrigerator (4 °C). Seeds held in room temperature (~30 °C) for 2–11 days survived and grew to seedlings. Survival of the seedlings in the field was also correlated with the performance of the seedling when raised for 10 weeks in the laboratory. Seedlings stored in refrigerator had reduced survival (87.5%) in the field compared to seedlings stored 2–5 days in room temperature (100% survival). However, seeds stored up to 8 days, even at room temperature, produced seedlings that did not survive well either in the laboratory or in the field (survival was less than 20%). Our results indicate that long-term seed storage in the laboratory will not be feasible for restoration. Because using seeds for restoration is important for maintaining genetic diversity, the establishment of a seedling nursery in the field provides a viable alternative for *Enhalus* restoration, although it will be logistically more challenging than a seed storage bank.

1. Introduction

Seagrass beds provide significant ecological functions in coastal ecosystem in maintaining coastal biodiversity and fisheries productivity. However, seagrasses have been declining globally due to anthropogenic effects (Orth et al., 2006a; Waycott et al., 2009).

Habitat protection and restoration are important for maintaining and improving the quality of seagrass habitat. Seagrass restoration has been implemented over the past two decades with various techniques and with varying degrees of success (Paling et al., 2009). Most seagrass restoration programs take clonal vegetative stock from the donor site and transplant it to the restoration site (Lee and Park, 2008; Biber et al., 2009; Paling et al., 2009). This method could disturb the donor seagrass bed, especially for large seagrasses and climax species such as *Enhalus acoroides* that take a long time to recover (Calumpong and Fonseca, 2001). In addition, transplantation techniques that solely use vegetative stocks can lead to reduced genetic variation (Williams and Davis, 1996; Williams and Orth, 1998). Genetic variation can enhance the resilience of the seagrass to stresses (Kendrick et al., 2012).

Seeds are rarely used in seagrass restoration (Fonseca et al., 1998). Orth et al. (2006b) stated that the use of seed should be prioritized in seagrass restoration programs in the future for several reasons: (1) many seagrass species produce seeds in large quantities that can be more easily harvested and potentially stored, (2) harvesting seeds is less destructive than taking adult plants from the donor site, and (3) seeds can lead to greater genetic diversity. On the other hand, seeds have a high failure rate to grow in nature due to the unsuitability of environmental conditions or seed predation (Orth et al., 2002, 2007; Ort et al., 2014; Nakaoka, 2002). One solution to this problem is to collect the seeds from the wild and maintain them in a more controlled and protected environment until they can be released back to the wild when they have developed leaves, roots, and rhizomes important for withstanding extreme environmental conditions and predators. One approach is to create nurseries for seagrass seeds to provide a year-round supply of seeds for seagrass restoration purposes.

E. acoroides is the climax seagrass species in the Indo-Pacific. Flowering and fruiting of this species takes place the whole year around (den Hartog, 1970), but can differ depending on geographic

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regions (Verheij and Erftemeijer, 1993; Fortes, 1990). According to Rollon et al. (2003), the production of male and female flowers at *Enhalus* takes 2 months, while the production of fruits takes 5 months to complete. The fruit and seed have positive buoyancy. An almost mature fruit that removed from the plant would remain floated for up to 7 days, before it releases the seeds (Lacap et al., 2002).

In natural circumstances, the fruit will remain attached to the plant while dehisce to release 8–14 seeds per fruit. The seeds would remain floating for up to 14 h (Lacap et al., 2002) until the veil of seed decayed or ruptured (personal observation). We observed that *Enhalus* actually has seeds that have negative buoyancy. The seed that has membranous coat was wrapped with a white-fleshy structure; contain air bubble trapped inside it. This structure would float the seed and gave the seed an effect of positive buoyancy. The seed would immediately sink if the wrapper decayed or deliberately or unintentionally ruptured.

Seeds of *E. acoroides* have no dormancy (Orth et al., 2006b; Kendrick et al., 2012). Some of those seeds, in some extent, already showed the emergence of plumule (personal observation); however, type of seed and germination strategy of *E. acoroides* has not previously been examined in detail. Germination without dormancy has been reported for *Phyllospadix*, *Amphibolis*, *Posidonia*, *Thalassodendron*, *Enhalus*, and *Thalassia* (den Hartog, 1970).

According to Farnsworth (2000), there are three types of seed, dormant, viviparous and recalcitrant, and by definition *E. acoroides* has recalcitrant seed. It means that the seed may germinate readily within the fruit or soon after the dehiscence (cryptic viviparous) (Calumpong and Dangan, 2003). On the other hand, viviparous is where the embryo grows sufficiently to emerge visibly from within the seed tissue before dispersal (Farnsworth, 2000). There are two seagrass genera having viviparous seed, *Amphibolis* and *Thalassodendron* (Kuo and Kirkman, 1990), where their viviparous seedling remain attached to the parent plant until one or more leaf cluster have formed. In addition, Farnsworth (2000) stated that the embryos of these viviparous and recalcitrant species cannot tolerate desiccation which creates challenges for storing and preserving them.

Because *E. acoroides* produces non-dormant, direct-developing seeds, long term seed storage conditions might not be a viable option. Currently, there is little to no available information on how long the seeds of *E. acoroides* can be stored while maintaining their viability. The aim of this study was to determine if temperature and length of storage of *E. acoroides* seeds would affect its viability.

2. Materials and methods

This study was conducted in Barranglompo Island, Spermonde Archipelago, South Sulawesi, Indonesia (5°03′S, 119°20′E). In this location, *E. acoroides* can flower and fruit throughout the year (Verheij and Erftemeijer, 1993), however, flower production mostly occurs between June and July (personal observation), and the fruits are mature 5 months later (Rollon et al., 2003). For this study, we collected the mature *Enhalus* fruits during the peak fruit production at our study site in late November 2011 and we harvested the fruits by detaching them from the flowering stalk. Although we assume that the fruits and their seeds were of the same age and cryptic vivipary was not a confounding issue in our experiment, they may have been of different ages. In any case, if they were different ages, they were distributed haphazardly among treatments.

We tested *Enhalus* seed viability and subsequent survival and growth at two different temperatures (room at ∼30 °C, refrigerator at 4 °C) and four different periods of storage of fruits (2, 5, 8, 11 days). We selected the two temperatures because they are the most convenient storage conditions for large scale seed restorations in Indonesia. More elaborate storage conditions are not feasible currently. We set the storage duration periods based on our observation that *Enhalus* seeds germinate immediately after being released from the fruit and we estimate that 11 days would be the longest time to transport fruit from a nursery area to the restoration site.

A total of 64 mature fruits (each containing 9–14 seeds) of the *E. acoroides* were collected from Barranglompo Island. The fruits were transported in a dry container to the laboratory within 2 h. Readily developing seeds of *Enhalus* might be desiccation sensitive; however, this transportation technique had no measurable influence on the result found. Eight intact fruits were stored at each treatment combination.

We used 192 transparent cylinder containers (8 cm diameter, 20 cm tall) filled with sea sand to a depth of 10 cm and placed in eight aquaria connected in a recirculation system for a week prior to starting the experiment. Temperature and salinity were maintained at 28 °C and 30 ppt respectively, by addition of freshwater. Each aquarium contained 24 containers and represented one treatment. Nitrate and phosphate concentrations of the seawater were measured before, in the middle, and at the end of the experiment.

2.1. Seed germination procedures

After storage, the seeds were removed from the fruits by hand. We carefully selected 24 seeds similar in size (avoiding the smallest and largest one) from each temperature-storage combination. Each seed was placed in a single container as described above; all containers were placed in one aquarium supplied with running seawater. We monitored germination as the first appearance of a leaf growing from a seed. Leaf length of subsequently germinated seedlings was measured every other day for a period of 10 weeks.

2.2. Planting seedlings in natural habitat

Seedlings that survived in the laboratory were transported to Barranglompo Island (origin of the seed) using a cool box filled with seawater without sediment. There were sufficient viable seedlings for five treatments: 2, 5, 8, 11 days storage at room temperature and 2 days at the cooler temperature. We planted three replicate frame plots for each treatment for a total of 15 frame plots. The plots were made from iron frame 50 cm × 50 cm × 20 cm containing 16 grids. The number of seedlings on each replicate differed due to different numbers of seedlings surviving each storage conditions: eight seedlings per replicate plot frame for 2 days-room temperature, eight per plot frame for 5 days-room temperature, four per plot frame for 8 days-room temperature, one per plot frame for 11 days-room temperature, and eight per plot frame for 2 days-refrigerator. The seedlings were tied to the cross sections of the gridded iron frame. The frame plots were deployed in areas with minimal seagrass vegetation with at least 2 m distance between plots and anchored to the substrate using iron stakes. For analyses, we averaged the leaf growth rate for each plot frame and calculated the percentage of seeds that survived of the total planted on each plot frame. The mean values for each plot were used in the analyses and figures. An exception was made for 11 days-room temperature treatment where there were only one seedling per plot available to be measured, therefore we could not have the mean from each plot for leaf growth rate. Consequently, we used value from the only one seedling data from each plot in the analyses for this treatment and it could also meet the sample size of three.

2.3. Seedling growth and field water quality

Measurements of leaf and root lengths of the seedlings were taken prior to planting in the field. Observations made in the field

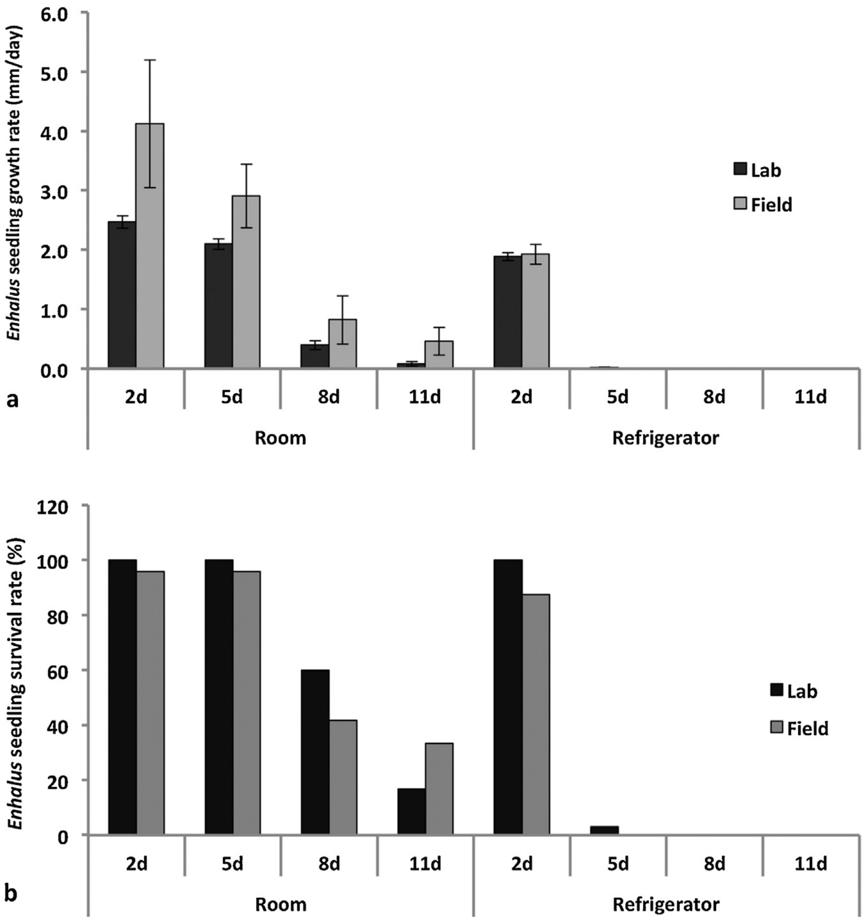

Fig. 1. (a) Average *Enhalus* seedling growth rate in the laboratory ($n = 24$) and planted seedling in the field ($n = 3$). (b) Average *Enhalus* seedling survival rate in the laboratory ($n = 24$) and planted seedling in the field ($n = 3$). Error bars represent standard errors.

included seedling survival and measurements of the length of the second leaf from each stand after 2 weeks and subsequently every week for 3 months.

Temperature and salinity were measured in situ during observation time using water quality checker (WQC-22A DKK-TOA Corp). Water samples from the water column were taken haphazardly from three plots for nitrate and phosphate initially, mid-way, and at the end of the study.

2.4. Data analyses

Comparison of the growth parameter and survival of *Enhalus* seedlings between treatments in the field was analyzed using one-way ANOVA. When the results were significantly different between treatments, a post hoc Tukey test was conducted (Sokal, 1981).

3. Results and discussion

3.1. Effects of storing fruits in the laboratory

Storage conditions of seeds of *E. acoroides* had a significant influence on seed survivorship and seedling growth. In the laboratory experiment, seeds from *Enhalus* fruit stored at room temperature (~30 °C) germinated and grew better than seeds from fruit that were refrigerated. The length of storage also affected seed germination and the growth of the seedlings. Maximum survival and seedling growth were found at seeds from fruit stored at room temperature for 2 days (Fig. 1a and b). All seeds stored at room temperature for up to 5 days germinated and grew >2 mm per day although growth rate from 2 days storage was higher. Seed viability (germination) and seedling growth are reduced the longer the seeds are stored, consistent with the lack of dormancy and cryptic vivipary in *E. acoroides* (den Hartog, 1970; Orth et al., 2000; Farnsworth, 2000; Calumpong and Dangan, 2003).

Seeds that were stored at 4 °C for more than 2 days turned black and failed to germinate. Placing the fruit in a refrigerator dehydrates the fruit and the seeds inside it causing them to turn black. Even though one seed stored for 5 days germinated, it stopped growing after 18 days. These results indicate that low temperature (refrigerator; 4 °C) would hamper and even damage the seagrass seeds, including the seed storage reserves. Stored reserves, especially in direct developing seeds such as *Enhalus* and *Posidonia*, is important for initial growth of seedling after germination (Balestri et al., 2009; Statton et al., 2013).

Parallel to the results on seedling growth, more seedlings survived from fruits stored at room temperature (70%; 67 of 96 total seeds) than at refrigerator (26%; 25 of 96 total seeds) and the survival decreased with the increased period of storage at both temperatures (Fig. 1b). Seed viability was maintained for a maximum period of two days when stored at 4 °C. The storage effect was stronger at the low temperature than in room temperature.

This study found that *Enhalus* seed germination and growth was adversely affected by length of storage and storage temperature. In addition to these factors, Dagapioso and Uy (2011) also reported negative effects of burial and desiccation on *Enhalus* seed germination and seedling development.

3.2. Performance of seedlings in the field

Salinity during field experiment was in optimal range for *E. acoroides* growth (30–31 ppt). Dissolved nutrients, both nitrate and phosphate, in the water column were also favorable for seagrass growth, which are 1.99–3.19 μg/L and 1.15–1.69 μg/L, respectively. Temperature ranged from 27 to 29 °C, which is within the range optimum for seagrass growth (Hemminga and Duarte, 2000).

Seedlings treated identically in terms of temperature-storage conditions in the laboratory exhibited significant variation in growth and survival in the field. The growth rate of seedlings varied significantly depending on how the fruits were stored in the laboratory ($F_{4,10} = 6.682$, $P < 0.01$). The growth rates of *Enhalus* seedlings from 2 and 5 days storage at room temperature and 2 days storage in the refrigerator were similar ($P > 0.05$), while longer storage of 8 and 11 days resulted in a lower growth rate compared to 2 days storage at room temperature ($P < 0.05$ and $P < 0.01$, respectively). There were no differences between 5 days versus 8 or 11 days storage at room temperature ($P > 0.05$ and $P > 0.05$, respectively) and also with 2 days storage in the refrigerator ($P > 0.05$) (Fig. 1a).

There was a significant difference in the survival rate of seedlings of *Enhalus acoroides* from different storage durations when grown in the field ($F_{4,10} = 3.733$, $P < 0.05$) (Fig. 1b). Survival rate of seedlings from 2 and 5 days storage at room temperature and 2 days storage in the refrigerator was also similar ($P > 0.05$ and $P > 0.05$, respectively), but they were different from 8 and 11 days storage in room temperature ($P < 0.05$ and $P < 0.05$, respectively).

The trends of seedling growth rate in the field followed the trend of seedling growth in the laboratory. Thus, the temperature and storage length in the laboratory were crucial for seedling growth in the field.

After 5 days storage in the laboratory, the seedlings were obviously smaller and the roots were short (3–59 mm for 8 days and 0–34 mm for 11 days). In contrast, storage duration of 2 and 5 days resulted in seedlings with longer roots (25–122 mm) which would provide for improved anchoring in the substrate and minimize the potential for uprooting by waves. Seedling size has also a great influence on seagrass growth, where larger seedlings produce larger plants than smaller seedlings (Glasby et al., 2015). The smaller size of the seedlings after longer storage is important because smaller individuals were more susceptible to animals that disturb the sediment or clip seagrass leaves, such as the callianassid shrimps (Stapel and Erftemeijer, 2000; Kneer et al., 2008) that we observed in our plot.

In conclusion, the temperature and duration of storage of *E. acoroides* fruits significantly affected the growth and survival of the seagrass seedlings. *Enhalus* fruits could be stored maximally up to five days at room temperature, after which the germinated seedlings will have high growth and survival if immediately planted. Field survival of seedlings, for example in a nursery, will be affected by bioturbators as well as the wave energy regime and substrate type (Lanuru, 2011; Rivers et al., 2011). Our results indicate that long-term seed storage in the laboratory will not be feasible for restoration. We recommend that it will be important to establish a seed nursery in the field for *Enhalus* restoration because it has no seed dormancy and does not tolerate long storage. This outcome supports Statton et al. (2013) for *Posidonia australis*, which also has no seed dormancy. Such a seed nursery will require habitat to be set aside for the nursery to provide a ready source of seedlings to harvest for restoration.

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