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Note

Germination of fresh and stored Texas wild rice seeds, an endangered aquatic macrophyte

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INTRODUCTION

Texas wild rice (*Zizania texana* Hitchc.; TWR) is a federal endangered plant endemic to the upper 4 km of the San Marcos River, Hays County, Texas (Poole et al. 2007). Because of its decline, limited location, and persistent threats, TWR was one of the first 11 plants listed as federally endangered (USFWS 1978).

TWR prefers swift-moving cool spring-fed runs and is most commonly found growing in coarse sandy soils at water depths less than 1 m (Poole and Bowles 1999). TWR is a perennial that thrives in faster currents, but will flower in shallower water and slower currents. Most emergent culms produce flowers that develop roots at their nodes to form tillers. These tillers either senesce or break off where they can lodge on debris or other aquatic plants and take root.

Threats to TWR include aquifer depletion, habitat destruction and alteration, invasive species, droughts, floods, recreational impacts, and its extremely small and limited range (Poole 2002). It is theorized that TWR may have existed in other spring-fed rivers in Central Texas, but all the other springs with the exception of the San Marcos River have dried up in the past (Probert and Longley 1989, Horne and Kahn 1997). Because of its decline, limited location, and persistent threats, TWR was one of the first 11 plants listed as federally endangered (USFWS 1978).

Historically, TWR in the San Marcos River seldom flowered and produced seeds (Emery 1977, Vaughn 1986). Observations of TWR from 1957 to 1978 revealed that no viable seed were being produced (Emery 1977). TWR is protogynous and wind-pollinated (Emery and Guy 1979), an indication that a large number of plants may have to be in proximity at different flowering stages for fertilization and seed production to occur (Power 1997).

A primary goal of the Edwards Aquifer Recovery Implementation Plan is to maintain, restore, and increase native aquatic vegetation in the San Marcos River, including the reintroduction of 7,500 m^2 of TWR over 15 yr (EARIP 2011). TWR stands that cover an area of at least 99 m^2 in the upper reaches of the San Marcos River appear to contain a level of genetic diversity adequate for use in the supplementation and maintenance of refugia populations (Richards et al. 2007, Wilson et al. 2017). Tillers are also used to maintain genetic clones of the plants in refugia at the San Marcos Aquatic Resources Center (SMARC; 29°50′23.9″N; 97°58′33.8″W). On the basis of the suggested area cover of a minimum of two TWR plants per square meter for EARIP (2011) restoration, a minimum of 15,000 plants will be needed. This number may outpace stem and tiller collection and utilization for *in situ* restoration efforts.

Seeds appear to be the most efficient and effective way to propagate and mass-produce TWR. Unfortunately, TWR seeds are only available seasonally and sporadically, and are recalcitrant and highly sensitive to drying (Horne and Kahn 2000). Seeds that remain viable under storage conditions are an important conservation tool because thousands of individuals can be maintained without regeneration (Walters 2004). TWR is a federally listed species, but protocols for mass propagation and seed storage are lacking. The development of a storage protocol in which seeds can be stored for a specific number of months and retain 50% viability would benefit the conservation of TWR.

The objectives of this study were to evaluate: 1) the temporal period for TWR to germinate from unrefrigerated, freshly collected seeds; 2) the germination of seeds refrigerated 0 to 12 mo using two different storage methods; and 3) the germination rates of refugia seeds previously stored under refrigerated conditions for 7 to 51 mo.

MATERIAL AND METHODS

Source of seeds

TWR seeds were collected from October 2009 to October 2015 from refugia plants maintained at the SMARC. Seeds were collected by gently pressing upward along mature panicles to dislodge seeds. Seeds were temporarily placed in a polyethylene bag with 10 ml of water to prevent desiccation. Depending on the experiment, seeds were 1) potted within 24 h of collection, 2) stored in a paper towel (25.5 × 23.5 cm) folded twice, hydrated with 10 ml of water, and sealed in polyethylene bag (towel/bag stored), or 3) stored in 50 ml of water in a sealed 75-ml glass jar (jar stored). Stored seeds were maintained under refrigerated conditions at 3 C for 1 to 51 mo. To simulate natural

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conditions, all seeds used in the experiments had pales and lemmas intact, and none was surfaced sterilized. Scarification of seeds was not required for germination.

Germination medium

Soil was comprised of a mix of topsoil (85%, 40–60% Houston Black clay), sand (5%), compost (5%), and wood chips (5%) purchased from a local nursery. The soil was saturated with Edwards Aquifer well water and placed in 1-L plastic pots. The seeds were pressed flat into the upper 0.1 mm of the soil and covered with 5-mm pea gravel. The pots were submerged to a depth of 0.25 m in 90-L tanks filled with Edward's Aquifer well water. Each pot was individually tagged with a numbered aluminum tag for identification. Plants were maintained in tanks with a water inflow of 11 L min^{-1} of well water at a water velocity of < 0.05 m s^{-1}. Water temperature and pH averaged 21.9 C (SE = 0.2) and 6.7 (SE = 0.03), respectively during the study.

Germination rates of fresh seeds

Fresh seeds were placed in water and potted within 24 h of collection as previously described. A total of 44 germination trials (225 seeds/trial, range 50 to 1,250) were evaluated from October 2012 to 2014. Germination was monitored weekly and germination rates were categorized as occurring within 1-mo intervals for 12 mo. Seeds were counted as germinated if the hypocotyl was observed to have broken through the seed coat. Percent germination was calculated by dividing the number of germinated seeds by the total number of seeds per pot.

Germination rates of stored seeds

All seeds for this experiment were maintained under refrigerated conditions at 3 C from 0 (control) to 12 mo. Towel/bag-stored seeds were hydrated with 10 ml of distilled water to standardized moisture content. Each month, the experiment was repeated by using a subsample of seeds ($n = 20$) for each storage method and potting as previously described. An additional study evaluated germination of seeds previously towel/bag stored and refrigerated at SMARC for 7 to 51 mo. Twenty seeds per treatment period (7 to 51 mo) were evaluated with four replications, and the experiment was repeated.

Statistical analysis

Descriptive statistics (and SE) were calculated for all data. Germination rates for freshly collected unrefrigerated seeds were analyzed with an ANOVA using Proc GLM procedure, and means separated using Tukey's test at the 5% level of probability in SAS software (version 9.2, SAS Institute, Cary, NC). Data were arcsine transformed to improve homogeneity of variance. Comparison of storage methods for each month were analyzed with t tests for differences at $P < 0.05$. Linear regression was used to calculate the germination inhibition (I_{50}) rate of storage methods. The I_{50} value is the value at which germination is less than or equal to 50%. Differences between germination rates for seeds 7 to 51 mo of age were analyzed with an ANOVA using Proc GLM procedure, and means separated using Tukey's test at the 5% level of probability.

RESULTS AND DISCUSSION

Germination rates of fresh seeds

The mean germination of freshly collected unrefrigerated TWR seeds was 73.6% (SE = 1.7). No significant difference ($P < 0.05$) was detected among the 44 seed germination trials using freshly collected seeds. Germination was significantly greater ($P < 0.05$) for fresh seeds at 2 mo compared with 1 and 3 mo, then declined from months 4 to 10 (Figure 1). Germination peaked at 2 mo at 26.6% and then declined. By 4 and 6 mo, 86.5% and 95.0% respectively of the seeds had germinated. Freshly collected seeds monitored for 11 to 12 mo did not germinate. On the basis of these results, germination trials should be monitored for a minimum of 6 mo to ensure that seeds capable of germination do so.

Germination rates of stored seeds

Seeds towel/bag-stored had significantly higher germination rates ($P < 0.05$) at 1, 2, 4, 5, and 6 mo compared with jar-stored seeds (Figure 2). Germination rates dropped below 50% at 6 mo for seeds towel/bag stored and 5 mo for seeds jar stored. Seeds towel/bag stored had germination rates at or above 40% for 8 to 10 mo of storage. The I_{50} germination rates were 6.8 mo ($y = -4.91x + 83.85$, $R^2 = 0.78$) and 4.1 mo ($y = -4.10x + 66.77$, $R^2 = 0.75$) for seeds towel/bag and jar stored, respectively.

For seeds towel/bag stored 7 to 51 mo, germination was significantly greater ($P < 0.05$) for seeds 7 to 9 mo of age compared with seeds greater than or equal to 10 mo of age (Figure 3). Germination rates ranged from 35% at 9 mo to 4.5% at 18 mo. No germination was documented for seeds stored 22 to 51 mo. Many packs of seeds stored over 8 mo were infected with a fungus or bacteria.

Results indicate that seeds can be used for propagation of TWR for restoration of habitat in the San Marcos River. Greater than 86% of unrefrigerated TWR seeds germinated within the first 4 mo of potting. TWR seedlings at 3 mo postpotting have an 88% survival rate (Jeffrey Hutchinson unpub. data). These results are similar to those obtained by Horne and Kahn (2000) in which germination of TWR declined after 2 mo and the overall germination rate was 67%. Collectively, these results suggest that approximately 65 TWR seedlings can be propagated for every 100 seeds potted.

The variation in germination by month for unrefrigerated seeds could be due to the time mature seeds remain attached to the panicle and the season of collection. Terrell et al. (1978) suggested that TWR seeds may require a short after-ripening period after obtaining germination rates of 60 to 100% for seed stored in water for 105 d at 3 C, which may explain the peak in germination at 60 d.

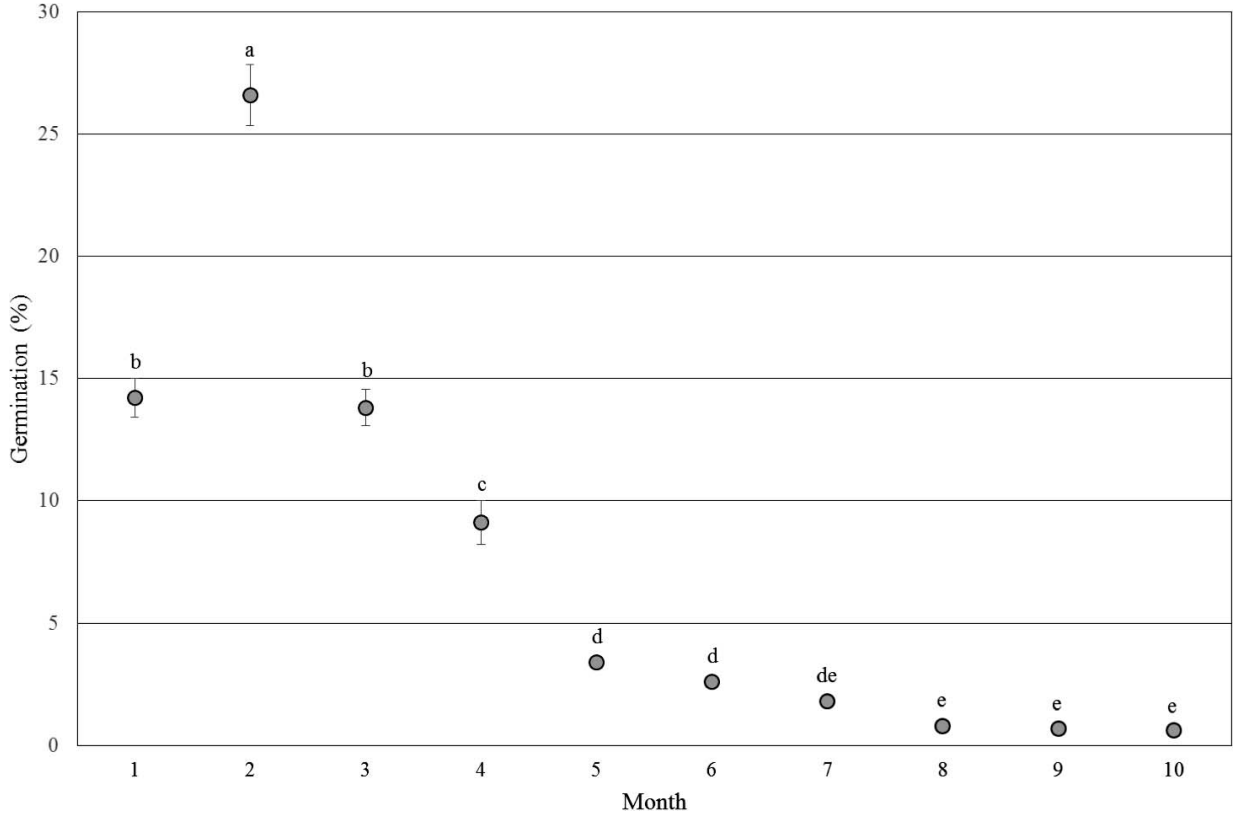

Figure 1. Mean percent germination per month for Texas wild rice seeds without refrigeration over a 10-mo period. Sample size was $n = 44$ for germination trials with a mean of 225 (SE = 32) seeds per sample. Different letters for months indicate significant differences at $P < 0.05$ on the basis of ANOVA and Tukey's multiple comparison test. Bars represent standard error of the mean for percent germination.

TWR seeds retain greater than 50% viability in towel/bag storage up to 6 mo under refrigerated conditions. Because the seeds are recalcitrant and based on the I_{50} value, storage under 3 C will maintain 50% seed viability for up to 6.8 mo. No sterilization of seeds was used in these studies and fungus was observed on many of the towel/bag-stored seeds. Walters et al. (2002) used 1.0% commercial sodium hypochlorite for 5 min to surface sterilize TWR embryos

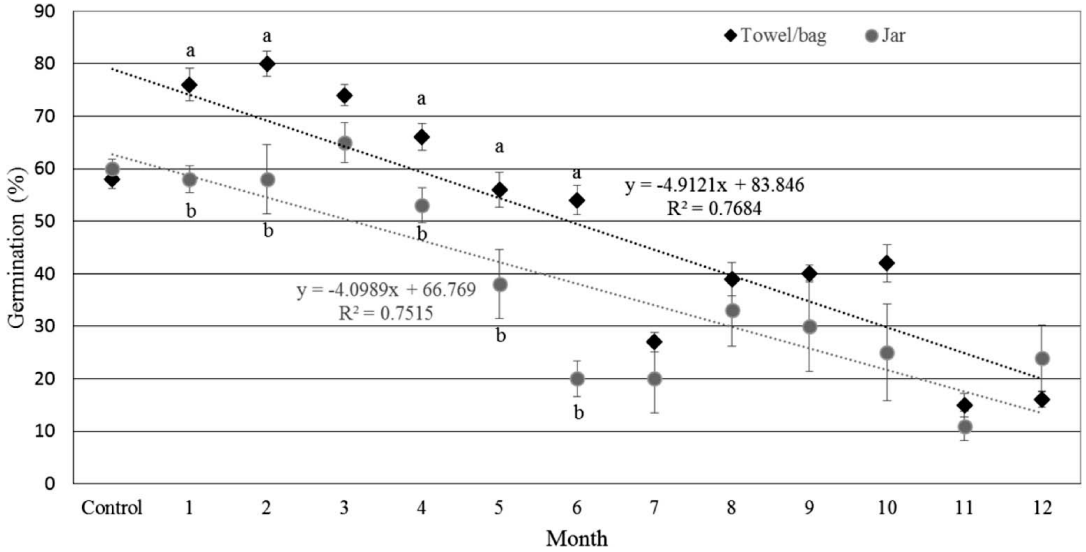

Figure 2. Mean percent germination of Texas wild rice seeds stored under refrigerated conditions for 0 to 12 mo for the towel/bag ( ) or jar (•) methods. Sample size was $n = 4$ germination trials with 20 seeds per trial. Different letters for germination by month indicate significant differences ($P < 0.05$) between storage methods on the basis of t test. Bars represent standard error of the mean for percent germination.

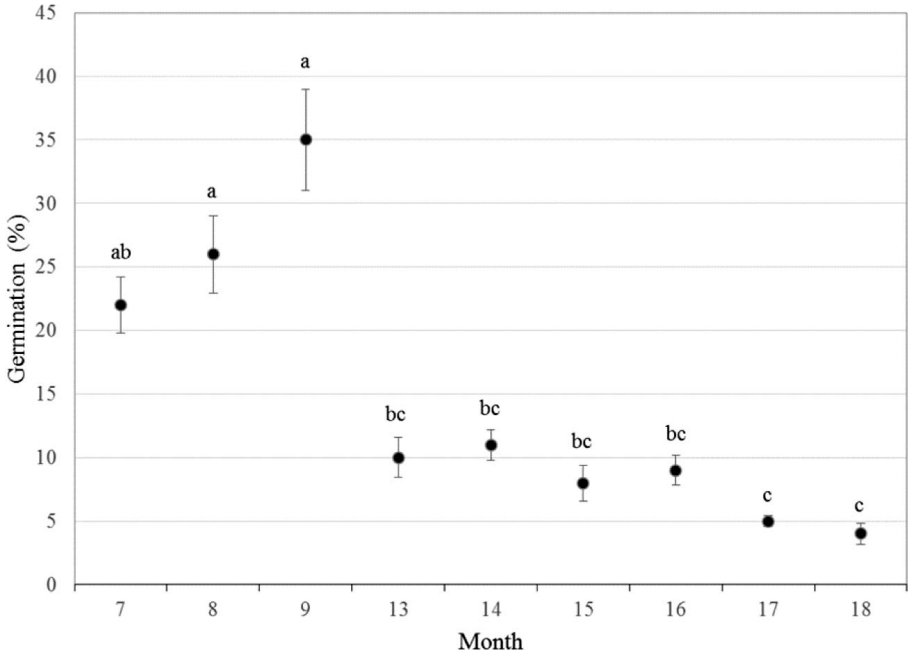

Figure 3. Mean percent germination of Texas wild rice refugia seeds stored with the towel/bag method under refrigerated conditions for 7 to 18 mo. Different letters for months indicated significant differences at $P < 0.05$ on the basis of ANOVA and Tukey's multiple comparison test. Bars represent standard error of the mean for percent germination.

without any apparent effects on germination. Controlling the growth of fungus and bacteria on stored seeds with sodium hypochlorite may result in an increase in the number of months that the seeds could be stored under refrigerated conditions. An additional problem with TWR seed storage is that some of the seeds stored for greater than 12 mo were completely dry. Determining the adequate amount of water to hydrate the paper towel to keep seeds from becoming dehydrated could also increase the storage of TWR seeds. Additional studies should examine sterilization methods to control fungus and bacterial growth on seeds stored in hydrated paper towels and water. Other methods such as storing seeds in vacuum-sealed plastic and partially drying seeds before storage under refrigerated conditions should also be evaluated. Cryopreservation techniques with supplemented sugar alcohols and sugars may offer techniques for long-term storage of TWR seeds (Walters et al. 2002).

TWR seeds in refugia should be maintained with subsets of seeds 0 to 6 mo of age to retain 50% viability. Seeds greater than 6 mo of age should be rotated out of refugia storage and used for propagation of plants for restoration efforts in the San Marcos River or supplementing experimental populations at SMARC. Supplemental seeds from refugia and wild stock can be collected and maintained in storage so that large numbers of viable TWR seeds are available for re-establishment of TWR in the San Marcos River if a catastrophic event destroys the only existing wild population. Maintaining a minimum of 9,000 seeds (1,500 seeds per month) for 6 mo in refrigerated conditions as refugia would result in a minimum of 4,000 seedlings if needed. Refugia stock of living plants combined with seed storage will provide the best insurance for the long-term survival of TWR.

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