Seed dormancy and germination traits of an endangered aquatic plant species, *Euryale ferox* Salisb. (Nymphaeaceae)

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ABSTRACT

Populations of *Euryale ferox* Salisb. have declined throughout its global range because of habitat loss and degradation. The present study aimed to evaluate the influence of storage temperature (4 and 20 °C), storage period (0, 90, 180, 270, and 360 days), light condition (light and darkness), germination temperature (constant 10, 15, 20, 25, and 30 °C), and seed size (two classes from 0.5 cm to less than 1.2 cm, and from 1.2 cm to less than 1.6 cm) on germination of *E. ferox* seeds. Our results indicated that seeds were dormant when shed and 4 °C stratification promoted germination more effectively than 20 °C. After stratification at 4 °C, the germination frequency of the larger seeds peaked at 90 days' stratification, whereas the smaller seeds maintained high germination frequencies up to 180 days' stratification. The different germination responses between smaller and larger seeds may reflect a difference in the rate of induction of dormancy in the annual dormancy cycle. Seeds germinated in both light and darkness, which demonstrated that light is not involved in the regulation of *E. ferox* seed germination. The optimal temperature for germination was 25 °C. Light condition × germination temperature interaction caused significantly higher germination frequency at 30 °C in light than in darkness, and the opposite trend at 15 °C.

Keywords: Nymphaeaceae, vulnerable, cold stratification, induction of dormancy, interaction of light and temperature

1. Introduction

Euryale ferox Salisb. (Nymphaeaceae) is a prickly annual aquatic herb with gigantic floating leaves 0.3–1.5 m in diameter. It is distributed from northwestern India to Japan and inhabits meso- and eutrophic water bodies such as lakes, ponds, reservoirs and rivers. In Japan, the species' distribution ranges from Kyushu to the northern part of Honshu, but habitat loss caused by drainage or land reclamation and water pollution has led to population decline and the species is classified as "vulnerable" in the Red List of Threatened Plants of Japan (Ministry of the Environment of Japan, 2012). Moreover, its populations have declined throughout its global range (Schneider et al., 2003).

It is well known that natural populations of *E. ferox* are subject to considerable annual variation in number of individuals (Miyashita, 1983; Kume, 1987). The species' seeds are believed capable of remaining dormant, even over several decades, when the external environment is unsuitable for germination (Wakita, 1959; Kadono, 1983; Ohtaki, 1987). This trait may influence annual variation in population sizes. However, seed dormancy and the germination characteristics of this species are poorly understood.

Seed dormancy is an adaptive mechanism to ensure that germination takes place in a suitable location and in suitable conditions (e.g., Baskin and Baskin, 2001; Fenner and Thompson, 2005). Temperature is regarded as the most important factor influencing seed dormancy (Bouwmeerster and

Karssen, 1992, 1993). Kumaki and Minami (1973) reported that about 30% of *E. ferox* seeds germinated after prechilling at 2–3 °C for 1 month, whereas at constant room temperature no seeds germinated. This result indicated that cold stratification breaks dormancy of the seeds. However, since the optimum period of stratification varies among species (Baskin and Baskin, 2001), and thus it is necessary to determine the optimal period of cold stratification for maximum germination and the influence of extended seed preservation on germination.

After release of seed dormancy, environmental factors such as temperature and light promote germination (Benvenuti et al., 2001; Penfield et al., 2005; Jha et al., 2010). A systematic quantitative study has not been conducted on the effect of temperature and light on germination of *E. ferox* seeds, but from field observations it is estimated that the seeds germinate between 20 and 25 °C and do not require light for germination (Okada, 1935; Wakita, 1959).

In addition to temperature and light, seed size may play an important role in seed germination for some species (Cideciyan and Malloch, 1982; Zammit and Zedler, 1990; Leverett and Jolls, 2013). Seeds of *E. ferox* are about 1 cm in diameter and their size varies among populations (Okada, 1928; Miyashita, 1983), among individuals in a population (Miyashita 1983; Hashimoto 1986), and even within the same individual (Hagiwara 1993). The phenomenon that small seeds of *E. ferox* require a shorter after-ripening period than large seeds has been observed (Okada 1935; Wakita 1959). However, a quantitative study of the relationship between seed size and germination has not been conducted.

The aim of the present research was to quantitatively verify seed dormancy and germination traits of *E. ferox* focusing on the influence of seed storage period, storage temperature, light condition, germination temperature, and seed size.

2. Materials and methods

2.1 Seed collection and storage

Fresh fruits and floating fresh seeds with arils were collected from late October to early November 2009 in the Hiranosawa pond, Kameoka, western Japan (35°04′03″ N, 135°33′55″ E). Hiranosawa pond consists of three small ponds with a total area of about 10 ha. It has been used as an agricultural reservoir. Genetic diversity of *E. ferox* is low within and among populations in Japan and the genotype of *E. ferox* in the Hiranosawa pond is the most widely distributed one in western Japan (the authors' unpublished data). Thus we considered that our samples are representative of the genetic diversity in this region. Seeds were separated from the fruits and the arils were removed in the laboratory. Small green- or skin-colored immature seeds were excluded.

The maximum diameter of collected seeds was measured with a digital caliper and seeds were divided into five size groups: from 0.5 cm to less than 1.0 cm; from 1.0 cm to less than 1.2 cm; from

1.2 cm to less than 1.4 cm; from 1.4 cm to less than 1.6 cm; and greater than 1.6 cm. The number of seeds in each size group was about 1,800 seeds, about 6,500 seeds, about 4,400 seeds, about 1,000 seeds, and 19 seeds, respectively.

Seeds of each size group were stored in separate plastic containers with four replicates wrapped in aluminum foil in order to avoid seed deterioration caused by fungal infection. The containers were filled with water and placed at either a constant low temperature (4 °C) to simulate the winter water temperature or a constant warm temperature (20 °C) to simulate the early summer water temperature in a small pond (Shimomura et al., 2010). Water was replaced regularly in complete darkness under a green safelight.

2.2 Germination tests

Germination tests were performed in light and darkness at constant temperatures of 10, 15, 20, 25, and 30 °C after 0, 90, 180, 270, and 360 days of stratification at 4 and 20 °C. Four replicates of 30 seeds each were placed in plastic containers and the seeds were submerged with water. The containers were placed in an incubator (TG-280CCFL-5LD, NKsystem, Osaka, Japan). On the basis of the number of collected seeds in each size group, four (from 0.5 cm to less than 1.0 cm), 14 (from 1.0 cm to less than 1.2 cm), 10 (from 1.2 cm to less than 1.4 cm) and two seeds (from 1.4 cm to less than 1.6 cm) per container were sown, respectively. Seeds of greater than 1.6 cm diameter were not used in germination tests because of the limited number of seeds available. We defined seeds from 0.5 to less than 1.2 cm diameter as small seeds and those from 1.2 to less than 1.6 cm diameter as large seeds.

In the light treatments, seeds were exposed to warm white fluorescent light providing photosynthetically active radiation of 20 μmol m^{-2} s^{-1} for 12 h day^{-1}. For the darkness treatments, all handling of containers was conducted in complete darkness under a green safelight and the containers were wrapped with aluminum foil after seeding.

For the light treatments, germination counts were made at intervals of 2–3 days for 60 days. For the darkness treatments, the containers were unwrapped at the end of the germination period (60 days), and the number of germinated seeds was counted. Seeds were recorded as germinated when the protruding radicle was $\geq$1 mm in length as defined by Okada (1935).

2.3 Data analysis

To examine the effects of light condition, germination temperature, and seed size on germination, we analyzed germination of seeds stratified at 4 °C for 90 days by constructing a generalized linear model with a binomial distribution with number of germinated seeds as the response variable and three factors (light condition, germination temperature excluding 10 °C, and seed size), three interactions of combinations of two factors (light condition × germination temperature excluding 10 °C, light condition × seed size, and germination temperature excluding 10 °C × seed size), and one interaction of the three factors (light condition × germination temperature excluding 10 °C × seed size) as explanation variables. We adopted the best model by stepwise selection. For the statistical analysis, we used the glm and stepAIC functions in R ver. 3.1.0 (R Core Team, 2014) and set the significance level as $p < 0.05$.

3. Results

Few seeds germinated immediately after harvest (no stratification) (Fig. 1). Regarding the seeds stratified at 4 °C, small seeds maintained high germination frequencies until at least 180 days (Fig. 1A and B). The germination frequency of large seeds was highest at 90 days and decreased at 180 days regardless of light condition (Fig. 1C and D).

With regard to the seeds stratified at 4 °C that germinated in light, the median germination time (i.e., the number of days until at least 50% germination frequency was attained in all of the four replicates within 60 days) was evaluated for the seven cases listed in Table 1. The shortest median germination time was 5.5 ± 0.5 days (mean ± SD) for small seeds stratified for 180 days and germinated at 25 °C.

Regarding the seeds stratified at 20 °C for 90 days, mean germination frequencies in light were

0, 0, 1.7 ± 1.9, 28.3 ± 7.9, and 29.2 ± 10.7 % (mean ± SD) at 10, 15, 20, 25, and 30 °C, respectively, and in darkness were 0, 0, 12.5 ± 7.4, 47.5 ± 18.9, and 18.3 ± 8.8 % at 10, 15, 20, 25, and 30 °C, respectively. After 180, 270 or 360 days of stratification at 20 °C, few or no seeds germinated.

The generalized linear model focusing on 4 °C stratification for 90 days revealed significant effects of light condition, germination temperature, seed size, light condition × germination temperature interaction, and germination temperature × seed size interaction (Table 2). The light condition × germination temperature interaction was significant owing to higher germination frequency at 30 °C in light (mean ± SD; 44.2 ± 6.9 %) than that in darkness (18.3 ± 12.3 %), and higher germination frequency at 15 °C in darkness (53.3 ± 9.8 %) than that in light (31.7 ± 9.8 %). The germination temperature × seed size interaction was significant owing to higher germination frequency of small seeds at 30 °C (37.5 ± 8.0 %) than that of large seeds (21.9 ± 12.3 %), and higher germination frequency of large seeds at 15 °C (49.0 ± 7.4 %) than that of small seeds (38.2 ± 4.6 %).

4. Discussion

Our results indicated that seeds of *E. ferox* are in a dormant state when shed and stratification for several months can break the dormancy (Fig. 1). Dormancy release was possible with 4 and 20 °C stratification, but was more strongly promoted by 4 °C stratification. With regard to seeds stratified at 4 °C, the germination frequency of large seeds peaked at 90 days (Fig. 1C and D), whereas small seeds maintained high germination frequencies until at least 180 days (Fig. 1A and B). It was hypothesized that non-germinated seeds did not lose viability but dormancy was re-induced because the embryos were white and hard when dissected after completion of the germination tests, although the induction of dormancy was not investigated. The different germination responses between small and large seeds may reflect a difference in the rate of induction of dormancy in annual dormancy cycle.

Most flowers of *E. ferox* are cleistogamous, but chasmogamous flowers are also produced (Okada and Otaya, 1930). In many species, germination responses differ between seeds from cleistogamous and chasmogamous flowers (e.g. Weiss, 1980; Trapp and Hendrix, 1988; Ferreira and Reinhardt, 1999). Kadono and Schneider (1987) reported that seeds of *E. ferox* from chasmogamous flowers are slightly larger than those from cleistogamous flowers because fruits from chasmogamous flowers contain significantly fewer seeds than cleistogamous flowers and seed size is inversely correlated to the total number of seeds in each fruit. Although more research is required, it is hypothesized that *E. ferox* may produce many small seeds, in which the rate of induction of dormancy is relatively slow, from cleistogamous flowers and fewer large seeds that show relatively rapid induction of dormancy from chasmogamous flowers.

Seeds of *E. ferox* germinated in both light and darkness (Fig. 1), which indicated that light is unimportant for regulation of germination in this species. It is believed that *E. ferox* can form a persistent seed bank (Wakita, 1959; Kadono, 1983; Otaki, 1987). A light requirement for germination is a common feature among species that form persistent seed banks (Pons 2000). However, germination in large-seeded species might be expected to be insensitive to light because seedlings from large seeds can emerge successfully from a much greater depth that light cannot penetrate (Pons, 2000; Milberg et al., 2000; Pearson et al., 2002; Fenner and Thompson, 2005). Seeds of *E. ferox* are large (about 1 cm in diameter), which may be a factor in their not requiring light for germination.

The present study quantitatively verified that the optimal temperature for germination was 25 °C because the median germination times at 25 °C were shorter than those at 20 °C (Table 1), and the germination frequency was high at both 20 and 25 °C (Fig. 1). In some species, light interacts with temperature to regulate seed germination (Baskin and Baskin, 2001). In the present study, the generalized linear model focusing on 4 °C stratification of 90 days revealed that light condition × germination temperature interaction significantly affected germination (Table 2). The significant interaction was due to higher germination frequencies at 30 °C in light than darkness and higher germination frequencies at 15 °C in darkness than in light. The interaction between light and temperature is considered to be beneficial for germination of *E. ferox*, as the seeds often sink to the bottom of water bodies. If the temperature around the seed is low even though light reaches the bottom of the water body, germination will be inhibited, which enables avoidance of an unsuitable external environment for growth. On the other hand, germination could occur at a low temperature when the seeds are buried at the bottom of a water body and do not receive light, which permits increased opportunity for germination close to the level that would be observed if the seeds were not covered by soil.

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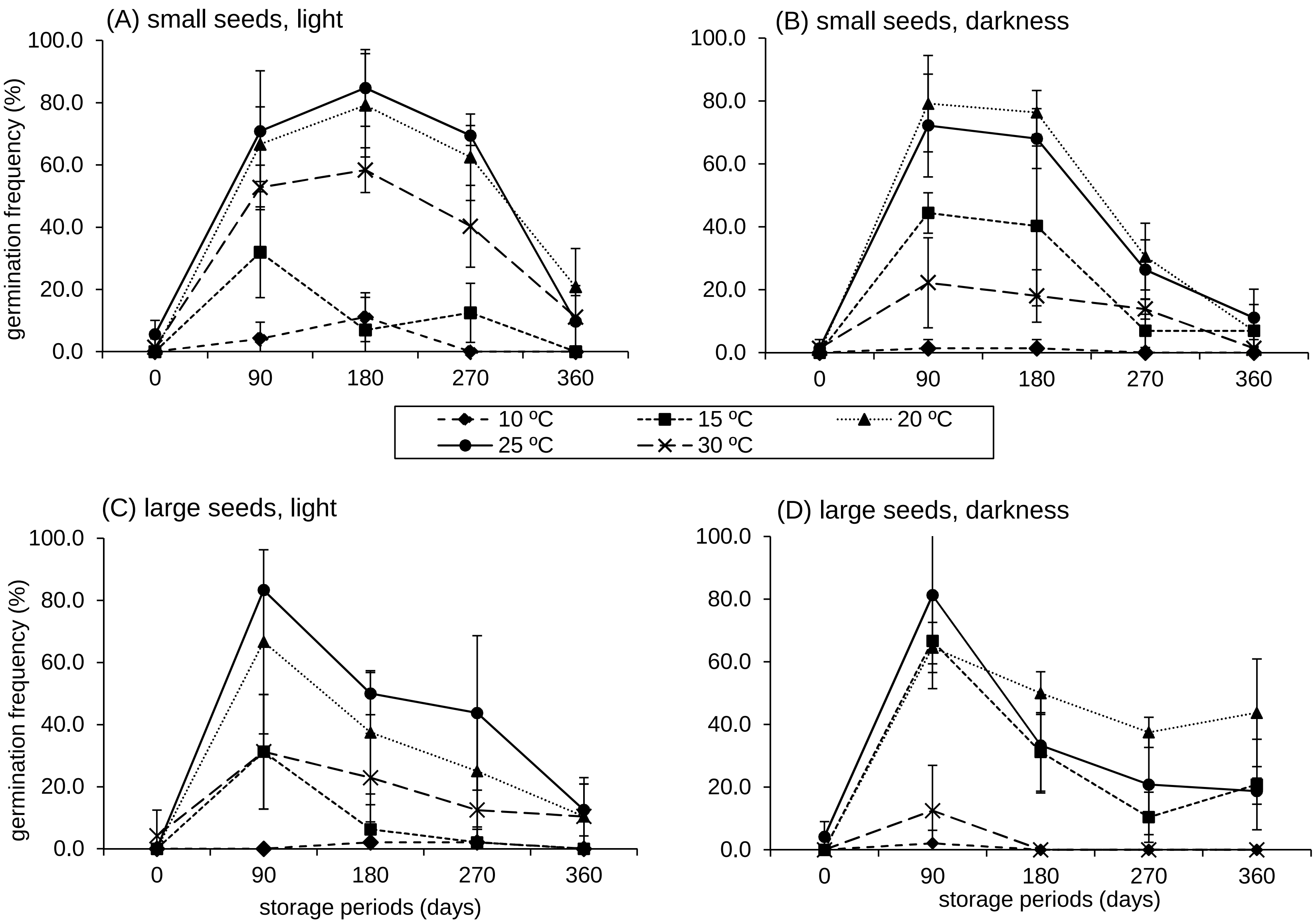
Figure captions

Fig. 1. Germination frequency of *E. ferox* seeds stratified at 4 °C and germinated at five constant temperatures ranging from 10 to 30 °C. (A) Small seeds germinated in light, (B) small seeds germinated in darkness, (C) large seeds germinated in light, and (D) large seeds germinated in darkness. Symbols and error bars represent the mean ± SD (n = 4).



(A) small seeds, light
(B) small seeds, darkness
(C) large seeds, light
(D) large seeds, darkness
germination frequency (%)
100.0
80.0
60.0
40.0
20.0
0.0
0
90
180
270
360
storage periods (days)
10 ºC
15 ºC
20 ºC
25 ºC
30 ºC

Table 1 Median germination time for *E. ferox* seeds, which is the days required for the germination frequency of all four replicates to reach at least 50% within the 60-day experimental period.

Germination temperature	Storage periods			
	90 days		180 days	270 days
	small seeds	large seeds	small seeds	small seeds
20 °C	20.8 ± 9.5	–	11.5 ± 6.1	–
25 °C	10.8 ± 2.6	8.5 ± 1.5	5.5 ± 0.5	14.5 ± 1.7
30 °C	–	–	8.0 ± 1.6	–

Values are the mean ± SD

"-" indicates that germination frequency of all of the four replicates did not reach 50% within the 60-day experimental period.

Table 2 Effects of germination temperature, light condition, and seed size on germination of *E. ferox* seeds

	Estimate	SE	*z* -value	*P*
Germination temperature	−0.12	0.02	−5.087	< 0.001
Light condition (in light)	−2.83	0.56	−5.081	< 0.001
Seed size (small)	−1.17	0.57	−2.048	0.04
Germination temperature × Light condition (in light)	0.12	0.02	5.19	< 0.001
Germination temperature × Seed size (small)	0.05	0.02	2.14	0.03
Intercept	2.86	0.54	5.29	< 0.001

AIC (selected model/null model) : 437.9 / 441.1

Models that have the highest power of explanation are shown.