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Seed ecology and pre-germinative treatments in *Magnolia schiedeana* Schlecht, an endangered species from Mexico

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Abstract

The life stages of dispersal and survival of seeds in soil are essential facets of demographic study. The successful pre-germination seed treatments are needed in order to support reintroduction programs and conservation of endangered species. This study encompassed seed survival at different depths and removal of *M. schiedeana*. Also we tested the efficacy of several pre-germinative treatments of *M. schiedeana* seeds. Seeds of *M. schiedeana* survived for two years in the soil, suggesting the ability to form a persistent seed bank. The viability of seeds, buried for two years in the soil at different depths (soil surface, 5, 10 and 15 cm), did not differ significantly ($P > 0.05$). With exposure to vertebrates, removal of seeds was 100% during the first month, whereas the exclusion of vertebrates resulted in removal of only 32% of seeds after one year ($\chi^2 = 13.75$, $P < 0.05$). The most successful pre-germinative treatments were 1) "48 h" treatment, where mechanically scarified seeds were placed in a layer of wet, sterilized river sand, incubated at 4-10°C for 13 days and subsequently soaked with sterile water for 48 h soaking seeds in water at room temperature (18±2°C), and 2) the "30°C" treatment, where mechanically scarified seeds placed in warm water (30°C) until the water had cooled to room temperature, and then soaked in sterile water for 48 h (both 84% germination). However, the "30°C" treatment had longer period of germination (35±2.3 days).

Key words: Cloud forest, germination, Mexico, mechanical scarification, seed bank, seed dormancy, seed dynamics, viability of seeds.

Introduction

Removal is the process which includes predation and movement of seeds [1]. Seeds are moved from one place to another by dispersing agents or predators, such as ants, birds, mammals, and also by abiotic factors, such as water, wind or gravity [2]. This ecological process is important in the reproductive cycle of many plants, especially those that have no dispersal agents [3, 4]. Seeds can also accumulate in the soil and form a temporary or persistent seed bank [5-7].

The seed bank forms a potential gene pool accumulated over time and is essential for the balance of demographic parameters for many species. It forms a part of the genetic diversity of the population and is the source of the last gene expression that can result from natural selection [8]. Many forest species are capable of establishing seed banks, which play an important role in forest regeneration [2]. The life stages of dispersal and survival of seeds in soil are essential facets of demographic study [5].

Seeds of the genus *Magnolia* exhibit low rates of germination [9]. It has been shown that the seeds of some species of this family have an exogenous dormancy (caused by oils and inhibitors of the sarcotesta and lignified testa, preventing the entry of water), e.g. *Magnolia grandiflora*, *M. acuminata*, *M. fraseri*, *M. virginiana* [9], *M. dealbata* [10] and *M. portoricensis* [11], while others, such as *M. iltisiana* [14], have an endogenous dormancy (caused by the testa exerting pressure on the embryo and thereby retarding germination and seedling growth). No studies of this type have been conducted for *M. schiedeana*.

In previous studies of the genus *Magnolia*, different pre-germinative treatments have been used, including: 1) imbibition in phytohormones and maceration [12]; 2) mechanical scarification and hot water [13]; 3) scarification, through the digestive tracts of birds [11]; 4) cold stratification 9, 10, 12-14; and 5) imbibitions in water [10, 13]. The most successful germination inducement treatments in *Magnolia* (e.g. *M. dealbata* and *M. iltisiana*) have been maceration, mechanical scarification, cold stratification and imbibition in water. However, the results among those treatments have been different (from 40 to 100% of germination in periods from 40 to 60 days) [9, 10, 12, 13]. Pre-germinative treatments are important for restoration and reintroduction programs of species with special conservation status (e.g. endangered species).

The Magnoliaceae family consists of 12 genera and approximately 220 species of evergreen or deciduous trees and shrubs native to Asia and America [15]. In Mexico, twelve species and two subspecies of the genus *Magnolia* have been recognized [16-19]. *Magnolia schiedeana* is endemic to Mexico [20, 21] and is considered threatened under the Norma Oficial Mexicana [22] and in danger of extinction, according to Cicuzza *et al.* [18]. *M. schiedeana* has a limited distribution, further exacerbated by the continuing destruction of their habitat [23, 24] and also by its limited seed production [25]. In this research we answer the following questions: 1) is the seed bank of *M. schiedeana* persistent or temporary, 2) are the vertebrates important to the seed removal from *M. schiedeana*

and 3) which pre-germination treatment is the most successful in inducing germination and a shorter germination time?

Materials and Methods

Study area and description of Magnolia schiedeana: Seed collection was conducted from two populations of *M. schiedeana*; one located in Banderilla ("Cerro de La Martinica"; 19°34'16.4"N and 96°56'14.88"W) and the other in Acajete ("Mesa de la Yerba"; 19°33'7.45"N and 97°01' 3.1"W). Both sites are located in the state of Veracruz, Mexico, and are situated at 1461 and 1864 m asl, respectively. At both locations, the climate is cold and humid, with average annual temperature and precipitation of 12-16 °C and 1350 - 1451mm, respectively [26].

Magnolia schiedeana has shown little morphophysiological change from the Tertiary period [27, 28], and is an evergreen tree of up to 25 m in height, with leathery and glabrous leaves [29]. Its white flowers, which are solitary and consist of 3 sepals and 6 petals, do not produce nectar and the stigmas are receptive for reproduction for only a short time. For successful reproduction to occur, the flowers of *M. schiedeana* require an animal vector for the transfer of pollen between flowers (xenogamous) [21, 25]. The fruit is polyfollicular and 4-8 cm long, the seeds are elliptic to obovate, 5 mm x 7 mm, with a soft and fleshy red sarcotesta, light brown testa, abundant endosperm and a straight and rudimentary embryo, with elliptical cotyledons [30]. Seeds are often preyed upon by squirrels [31].

Magnolia schiedeana is associated with tropical montane cloud forest species such as *Carpinus caroliniana* Walt, *Clethra mexicana* DC, *Eupatorium daleoides* (DC) Hemsley and *Fagus grandifolia* var. *mexicana* Martínez, among others [32].

Seed collection: Due to the weak fruiting of *Magnolia schiedeana*, the polyfollicles mature in the two populations were collected. The population of the "Cerro de La Martinica" contributed 94% of polyfollicles collected, while the rest were from "Mesa de la Yerba". All polyfollicles were placed and mixed on a table at room temperature in the shade (ambient aeration). From 1 to 10 days, the polyfollicles exposed their seeds and these were extracted manually.

Because the suitable seeds are scarce, two samples of 25 seeds from collection were taken to assess seed viability with a solution with 2,3,5-triphenyltetrazolium chloride (0.01% solution in phosphate buffer pH 7) [33].

Seed bank: Six hundred seeds with sarcotesta intact were subjected to four treatments, comprising six replicates, each of 25 seeds. The treatments were: 1) seeds placed directly on the soil surface, 2) seeds buried at 5 cm depth, 3) seeds buried at 10 cm depth and 4) seeds buried at 15 cm depth. To avoid seed loss or inadvertent mixing between treatments, each replicate (25 seeds) was placed within a mesh bag (10 cm x 10 cm with a 2 mm mesh), labeled with aluminium foil (7.5 cm x 2 cm). Six plots of 1 m^2 were chosen at random in the understorey of the tropical montane cloud forest of "Cerro de La Martinica". In each of the quadrats, seeds were subjected to four treatments. Three replicates per treatment were exposed for one year, while the other three replicates were exposed for two years (fifth treatment). At the end of each year, the seeds were extracted and tested for viability with 0.01% tetrazolium chloride [33]. The numbers of viable seeds in each treatment (values transformed to ranks), the years and interaction were analyzed using the GLM process [34].

Seed removal: One hundred and twenty seeds, with sarcotesta intact, were subjected to two treatments comprising three replicates, each of 20 seeds. The treatments were: 1) seeds exposed to small vertebrates by placing them on an aluminium mesh tray 15 cm x 15 cm wide and 2 cm high with 0.5 cm mesh, and exposing them to removal on the soil surface, and 2) seeds isolated from small vertebrates by enclosing them in an aluminium mesh box (15 cm x 15 cm x 5 cm with 0.5 cm mesh).

Treatments were randomly placed in the tropical montane cloud forest of "Cerro de la Martinica". The metal boxes and trays were lightly covered with soil and leaves and fixed in place with two nails (4 cm) each. We recorded the number of seeds removed each month for one year and the final results were analyzed using a χ^2 test [35]. Surviving seeds were then tested for viability with tetrazolium chloride to 0.01% [33].

Pre-germinative treatments: To determine the most successful pre-germinative treatment for *M. schiedeana*, we used all of the available seeds (n = 775) to do the following experiments, consisting of five treatments with five replicates of 25 healthy seeds each. The best pre-germinative treatments found in other research were used in this experiment [9-14, 36]. The treatments used in this research were: (i) the "SWS" treatment, i.e. the control, where seeds with intact sarcotesta were germinated immediately after collection, (ii) the "SMS" treatment, where mechanically scarified seeds (sarcotesta removed manually) were rinsed with sterile water and germinated immediately; (iii) the "24 h" treatment, where mechanically scarified seeds were placed in a layer of wet, sterilized river sand, incubated at 4-10°C for 13 days and subsequently soaked with sterile water for 24 h; (iv) the "48 h" treatment, where mechanically scarified seeds were placed in a layer of wet, sterilized river sand, incubated at 4-10°C for 13 days and subsequently soaked with sterile water for 48 h; (v) The "30°C" treatment, where mechanically scarified seeds placed in warm water (30°C) until the water had cooled to room temperature, and then soaked in sterile water for 48 h, modified from Saldaña *et al.* [12] (without sarcotesta). Water was changed every 12 h when seeds were soaked.

After applying the treatments, seeds were placed in Petri dishes with sterilised moist soil (through autoclaving process at 121°C for 15 min and 103 kPa) collected from the tropical montane cloud forest and left to germinate for 60 days at room temperature (18± 2°C). Seeds were considered to be germinated upon emergence of the radicle [10].

The effect of the treatments on germination was assessed by one way ANOVA, with values transformed to ranks and compared using a Tukey's test [35]. The period of germination (number of days required to germinate each seed) was compared between treatments using one-way generalised linear models (GLM), and multiple comparisons were performed using the Tukey's test [34]. The germination period observations were 24 h each. Finally, seeds that did not germinate after 60 days were tested for viability with tetrazolium chloride [33]. Live seeds per treatment were compared using the GLM process, with values transformed to ranks and a Tukey's test used for multiple comparisons [34].

Results

Seed bank: Viability of seeds collected was found to be 56%. During the study two replicates in the treatment consisting of seeds placed on the soil surface were lost (one replicate per year), and a replicate in each of treatment depths 5 cm and 15 cm was lost in the second year. Due to these replicate losses, we used the GLM of SAS [35]. Viability of seeds buried at different depths in the soil for two years was 9.1±0.4%. No significant differences were found between treatments (depth of burial), years or interaction between year and treatment ($P > 0.05$) (Fig. 1).

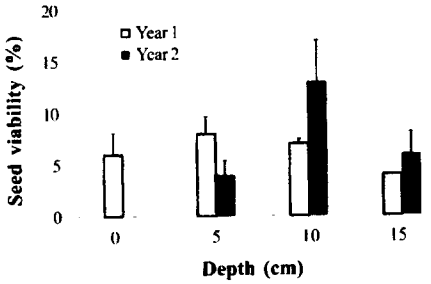

Figure 1. Percentage of viable seeds of *Magnolia schiedeana* after one and two years of burial. Bars ± standard deviation of the mean (SD). No significant differences were found between treatments (depth of burial), years or interaction between year and treatment ($P > 0.05$). Treatments are: Seeds placed on the surface, seeds buried at 5 cm depth, seeds buried at 10 cm depth and seeds buried at 15 cm depth.

Seed removal: Removal of seeds in the treatment exposed to small vertebrates was 100% during the first month. In the treatment consisting of seeds isolated from vertebrates, it was observed that they were invaded by fungi of the genus *Xylaria* sp. and small invertebrates (e.g. ants) in the first month, which caused the loss of sarcotesta in all the seeds. A 22% germination rate was subsequently recorded in the second month. The annual rate of removal by invertebrates was 32.5%, with a total of 18 seeds remaining at the end of the year (8% viable). Removal of seeds was significantly higher in the treatment with exposure ($\chi^2 = 13.75, P < 0.05$) (Fig. 2).

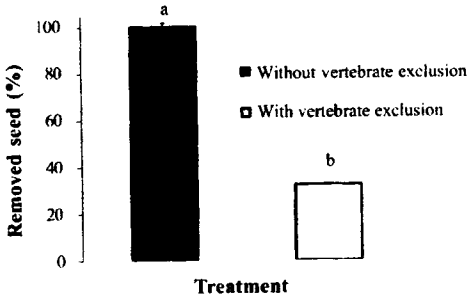

Figure 2. Percentage of annual seed removal of *Magnolia schiedeana*. Bars ± standard deviation of the mean. Treatments were significantly different ($P < 0.05$).

Seed viability and germination: Viability of all seeds collected was found to be 80%. The seed germination was found to significantly differ among treatments ($P < 0.05$). The most successful pre-germination treatments were 1) the "30°C" treatment and 2) the "48 h" treatment, both 84% germination. The least successful treatment was the control (SWS), in which no germination occurred (Fig. 3).

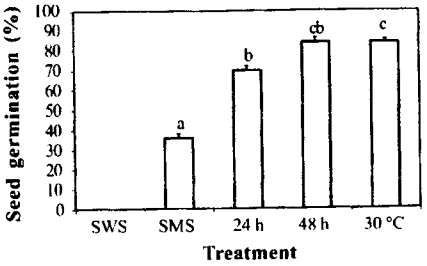

Figure 3. Percentage of germination in seeds of *Magnolia schiedeana* in response to treatments. Bars indicate mean ± standard deviation. Bars connected by the same letters indicate no significant differences ($P < 0.05$). Treatments are seeds with sarcotesta (SWS), mechanical scarification (SMS), mechanical scarification and stratified temperature 4-10°C for 13 days and soaking for 24 h (24 h), mechanical scarification and stratified temperature 4-10°C for 13 days and soaking for 48 h (48 h) and mechanical scarification with imbibition in water at 30°C and soaking for 48 h (30°C).

Effects of treatments in time of germination: Time of germination differed significantly among treatments ($P < 0.05$). The SMS, "24 h" and "48h" treatments produced the shorter period of germination (28- 31 days) while the "30°C" treatment registered the longer germination period (35±2.3 days) (Fig. 4). The SWS treatment did not germinate any seeds. After the 60-day period of the experiment, seeds that had not germinated were tested for viability, and no significant difference was found among treatments ($F = 0.28$, $df = 4$, $P = 0.88$).

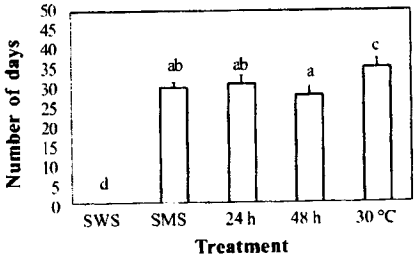

Figure 4. Average germination time (days) of seeds of *Magnolia schiedeana*. Bars indicate mean ± standard deviation (SD). Bars connected by the same letters indicate no significant differences ($P < 0.05$). The abbreviations for each treatment are shown in Fig. 3.

Discussion

Seed survivorship: Seeds of *M. schiedeana* can survive two years buried in the soil, which suggests that this species is capable of forming a persistent seed bank according to the definition from Fenner and Thompson [2]. Other species of *Magnolia* also have this capacity; for example, *M. dealbata* [13] and *M. stellata* [37]. The formation of persistent seed banks is typical of boreal species [38]. *Magnolia schiedeana* is a species with boreal affinities [39], and is cold-tolerant [40]. In general, dormancy in seeds of some species of the genus *Magnolia*, e.g. *Magnolia grandiflora*, *M. acuminata*, *M. fraseri* and *M. virginiana*, has been previously demonstrated [9, 10, 12, 13, 36]. Unlike seeds of *M. dealbata* [13], depth of burial of seeds of *M. schiedeana* had no effect on their viability.

However, in studies of *in situ* seed banks, Magnolia seeds have not been found. For example, Williams [41] found no Magnolia seed in soil taken from 5 cm depth in four tropical montane cloud forests in Mexico, despite the fact that *M. schiedeana* was recorded as the dominant canopy tree in one of the sites studied (Biosphere Reserve "El Cielo" Tamaulipas). Likewise, Álvarez-Aquino *et al.* [42] also found no seeds of *M. schiedeana* in six tropical montane cloud forest sites in Mexico, despite this species being present at two of the sites. It is possible, however, that these authors failed to find magnolia seed because the soil samples were relatively small and the predation of seeds is very high in these sites.

Seeds of *M. schiedeana* showed 100% removal during the first month in the treatment allowing exposure to vertebrates. Seed removal was possibly carried out by rodents or ants, as has already been reported for species of the genus *Magnolia*, such as *M. portoricensis* and *M. dealbata* [11, 13]. However, for *M. dealbata*, 100% seed removal took a period of eight months [13]. In the treatment isolating seeds from vertebrates, those of *M. schiedeana* showed 32.5% removal over one year, in contrast to *M. dealbata* which presented a constant removal of 99.5% for the same treatment [13]. In this treatment, by the second month after degradation of the sarcotesta by fungus or perhaps small invertebrates, 22% of the seeds of *M. schiedeana* had germinated. These results suggest that between 45.5% and 100% of *M. schiedeana* seeds could be removed by vertebrates and the burial of seeds by vertebrate trampling or litterfall, can help seeds to escape predation and thus contribute to the development of a persistent seed bank.

Further study on the fate of seeds of *M. schiedeana* is required in order to fully evaluate the role of removers on predation or dispersal of seeds. This information will be essential to generate models of the population dynamics [43] of this endangered species[44].

In short *M. schiedeana* has the ability to form a persistent seed bank. Seed removal by vertebrates is very high, from 45.5 to 100%.

Seed pre-germinative treatment: Germination was not induced in the pre-germinative treatments that left the sarcotesta intact. This result suggests that the sarcotesta promoted dormancy, as has been found in other species such as *M. dealbata* and *M. iltisiana* [10, 12].

Olson *et al.* [9] recommended breaking the dormancy of *Magnolia* seeds with cold stratification (0 to 8°C) for a period of 3 to 6 months (e.g. *Magnolia grandiflora* takes 40 days on average). However, our results for *M. schiedeana* show that dormancy is broken by stratification at 4-10°C in only 13 days, which is the same as the stratification used for *Magnolia dealbata* [13]. Following dormancy, seeds of *M. schiedeana* germinate successfully after soaking for only 48 h, or by mechanical scarification and imbibition at 30°C. This difference may be because both *Magnolia schiedeana* and *M. dealbata* are endemic to Mexico, where the climate is warm, while other magnolias (eg. *M. grandiflora*) are adapted to colder climates [38].

Regarding germination time, Saldaña *et al.* [12] reported that removing the sarcotesta of *Magnolia iltisiana* seeds followed by imbibition in water at 30°C resulted in a germination time of 60 days. However, in our study, the average number of days required to germinate seeds of *M. schiedeana* was only 28. The stratification treatment to seed without sarcotesta was not used for Saldaña *et al.* [12], maybe by using this treatment they could had a shorter period of germination.

Conclusions

The most successful pre-germinative treatment were "48 h" treatment, where mechanically scarified seeds were placed in a layer of wet, sterilized river sand, incubated at 4-10°C for 13 days and subsequently soaked with sterile water for 48 h soaking seeds in water at room temperature (18±2°C) for 48 h, and the "30°C" treatment, where mechanically scarified seeds placed in warm water (30°C) until the water had cooled to room temperature, and then soaked in sterile water for 48 h (both 84% germination). However, the "30°C" treatment had longer period of germination (35± 2.3 days).

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