

EXPERIMENTAL SEED GERMINATION FOR *EX SITU* CONSERVATION OF MEXICAN PODOSTEMACEAE

GERMINACIÓN EXPERIMENTAL DE SEMILLAS PARA LA CONSERVACIÓN *EX SITU* DE PODOSTEMÁCEAS MEXICANAS

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Abstract

Background: Podostemaceae are extremely susceptible to local extinction by habitat loss. Since ~70 % of the river systems in Mexico show some degree of water contamination, it is relevant to generate information about seed storage and germination behavior to design germplasm conservation strategies (e.g., *ex situ* seed banks) of Mexican podostemads.

Hypotheses: Seed germination decreases as seed storage time increases. Further, light quality, temperature and collection site influence similarly the germination response of both species.

Studied species: *Marathrum foeniculaceum* Humb. & Bonpl., *Noveloa coulteriana* (Tul.) C. Philbrick

Study site and years of study: 13 different seeds collections (1996-2013), at four locations in the rivers Horcones and Arroyo del Rincon Jalisco, México.

Methods: A germination chamber experiment was performed to evaluate the effect of temperature, light quality and storage time on the germination of both species.

Results: Seeds lose viability after nine years of storage. In both species, the time to reach the highest Accumulated Germination Percentage (AGP) was faster in seeds of one-two yr compared to seeds of six-seven yr. *N. coulteriana* have significant differences in Final Germination Percentage (FGP) between collection rivers. Storage time affects AGP of *N. coulteriana* more than in *M. foeniculaceum*.

Conclusions: Both species can form *ex situ* seed banks up to 8 yr age in paper bags storage. *N. coulteriana* is more susceptible to variation in storage conditions compared to *M. foeniculaceum*. Collection site affect seed germination after storing. Long-term conventional *ex situ* seed germination storage is not a viable strategy to conserve germplasm of Podostemaceae.

Keywords: germplasm, *Marathrum foeniculaceum*, *Noveloa coulteriana*, seed bank.

Resumen

Antecedentes: Las Podostemaceae son susceptibles a la extinción local por degradación de los ríos en que habitan. ~70 % de los sistemas fluviales de México están contaminados. Es relevante generar información del almacenamiento y germinación de semillas para diseñar estrategias de conservación *ex situ* de su germoplasma.

Hipótesis: La germinación de las semillas disminuye conforme aumenta el tiempo de almacenamiento. Además, la temperatura, la calidad de la luz y el lugar de colecta influyen similarmente en la respuesta germinativa de ambas especies

Especies estudiadas: *Marathrum foeniculaceum* Humb. & Bonpl., *Noveloa coulteriana* (Tul.) C. Philbrick

Lugar de estudio y años de estudio: 13 colecciones de semillas (1996-2013) en cuatro localidades de dos ríos de Puerto Vallarta, Jalisco, México.

Métodos: Evaluamos el efecto de la temperatura, calidad de luz y tiempo de almacenamiento sobre el porcentaje de germinación acumulado (PGA) y el porcentaje de germinación final (PFG).

Resultados: Las semillas pierden viabilidad después de nueve años de almacenamiento. En ambas especies el máximo PGA se alcanzó más rápido en semillas de uno-dos años. *N. coulteriana* mostró diferencias significativas en PFG entre ríos y el tiempo de almacenamiento afectó más su PGA.

Conclusiones: Ambas especies forman bancos de semillas *ex situ* de hasta 8 años (almacenadas a temperatura ambiente). *N. coulteriana* es más susceptible a la variación de las condiciones de almacenamiento que *M. foeniculaceum*. El sitio de colecta afecta la germinación de semillas almacenadas. El almacenamiento convencional no es una estrategia viable para conservar el germoplasma de Podostemaceae a largo plazo.

Palabras clave: banco de semillas, germoplasma, *Marathrum foeniculaceum*, *Noveloa coulteriana*

Freshwater ecosystems are subject of severe impacts by human activities like industrial, agricultural or urban discharges. This promotes habitat loss, threatening the biodiversity of these ecosystems (Ojeda 2011). Biodiversity loss in fresh-water ecosystems is even greater than in the most disturbed terrestrial ecosystems (Sala *et al.* 2000, Dudgeon *et al.* 2006, Vörösmarty *et al.* 2010). This is even worse in tropical regions, where most of biodiversity is located (Mittermeier *et al.* 1998, Bawa *et al.* 2004). In the face of the loss of thousands of threatened plant species it is fundamental to promote assertive conservation programs. This need has been recognized by the 10-year Global Strategy for Plant Conservation, which established the goal of preserve at least 75 % of threatened plant species worldwide through seed-based

in *ex situ* collections (Sharrock 2012). Thus, generating information of seed storage and seed germination is helpful to design effective strategies for germplasm conservation such as *ex situ* seed banks.

Podostemaceae family is the largest group of strictly aquatic angiosperms, which inhabit oligotrophic rivers in tropical and subtropical regions of the world. Podostemad life cycle is closely associated with the seasonality of the rivers they inhabit. During the rainy season when the water level is high, vegetative structures grow submerged and anchored to bedrock; later, when the water level decreases during the dry season, reproductive structures emerge (Figure 1A). Then flowering and pollination occurs, and fruits and seeds are formed; later, these seeds will reestablish the population

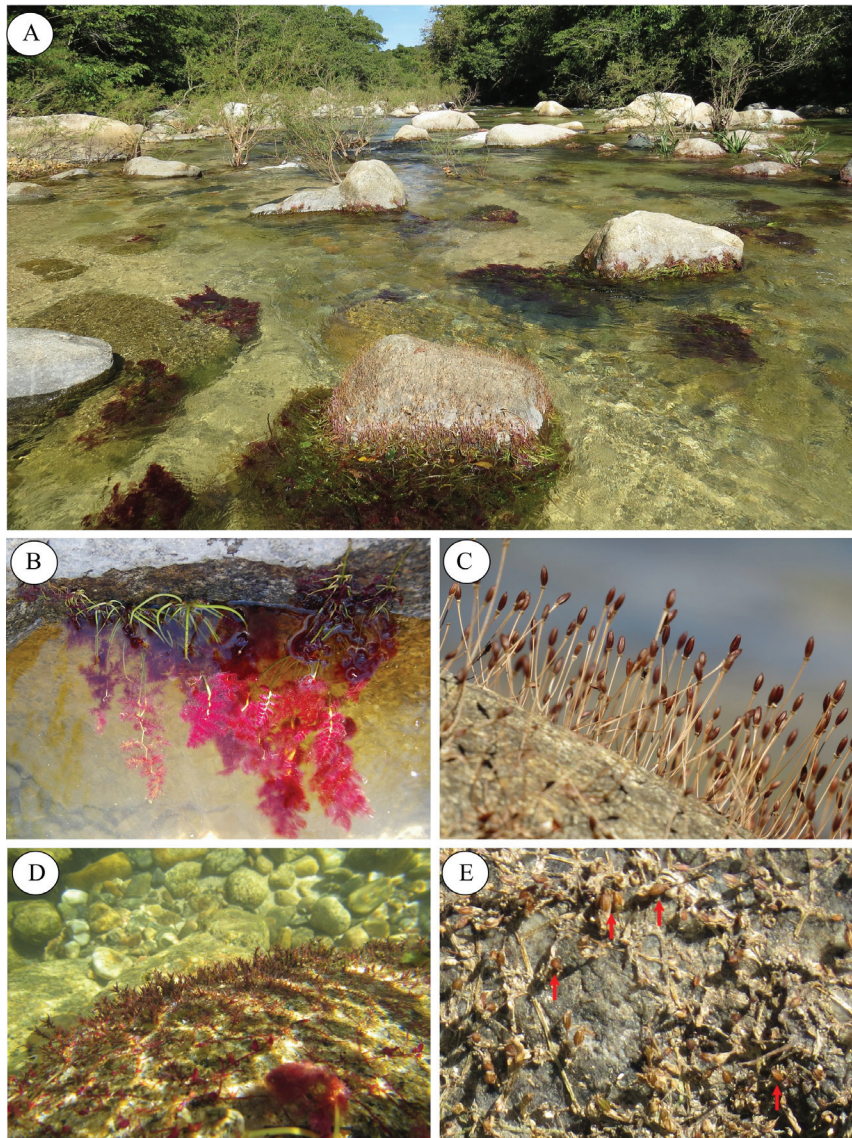


Figure 1. A) Characteristics of the river where podostemads fruits were collected. B) *Marathrum foeniculaceum* in vegetative phase. C) Fruits of *M. foeniculaceum*. D) *Noveloa coulteriana* in vegetative phase. E) Fruits of *N. coulteriana* (marked with red arrows).

during the next rainy season (Castillo *et al.* 2013). Podostemaceae seeds bear a mucilaginous seed coat, derived from the outer integument of the ovule (Philbrick & Novelo 1997). When seeds are released from the capsule, the cells of the outer integument are dry and collapsed. These cells hydrate rapidly when moistened after the first rain of monsoon, expand and become mucilaginous and sticky. This mucilage is a common feature in Podostemaceae that is involved in plant fixation to the substrate (Philbrick & Novelo 1997, Reyes-Ortega *et al.* 2009). Unlike most aquatic plants, sexual reproduction is the main way of reproduction of podostemads. Because of this, seed germination is a fundamental process in Podostemaceae, as it drives their distribution, survival, and abundance (Philbrick & Novelo 1995, 1997, Luna *et al.* 2012). Moreover, approximately 33 % of neotropical species of the family are single-river or two-river endemics (Cook & Rutishauser 2007, Philbrick *et al.* 2010, Katayama *et al.* 2016). This makes podostemads extremely susceptible to local extinction caused by habitat loss as a result of dams construction or water pollution through runoff of nutrients used in crop fields (Philbrick *et al.* 2010, Masahiro & Lansdown 2012).

Five genera of podostemads are distributed in México: *Marathrum*, *Podostemum*, *Tristicha*, *Noveloa* and *Vanroyenella*, containing seven species of which *M. plumosa*, *N. longifolia* and *N. coulteriana* are endemic to Mexico (Novelo & Philbrick 1997, Novelo *et al.* 2009, Tippery *et al.* 2011). *N. coulteriana* is considered a protected species by Mexican laws (SEMARNAT 2010). Considering that about 70 % of the river systems in Mexico show some degree of perturbation (Quiroz *et al.* 1997, Pérez *et al.* 2010, Vörösmarty *et al.* 2010), it is of high importance to generate information about podostemads seed storage and seed germination behavior. This knowledge will be extremely valuable to design strategies for germplasm conservation strategies like *ex situ* seed banks.

Seed germination is regulated by different external factors like light quality or temperature. Seed response to light is strongly regulated by the phytochrome (Pr/Pfr), which

is a red (R) and far red light (FR) photoreceptor in plants (Sawada *et al.* 2008, Kami *et al.* 2010). Absorption of red light (± 660 nm) converts the inactive form of the phytochrome Pr to its active form Pfr (promoting or inhibiting germination), whereas the absorption of far red light (± 730 nm) converts Pfr back to Pr. According to their response to light, seeds can be classified as positive photoblastic if light activates germination or negative photoblastic if light hinders germination. If germination is not related with light, seeds are considered non-photoblastic. Temperature can also influence the endogenous chemical mechanisms of germination (*e.g.*, ABA/GA hormone balance, phytochrome activity), modifying germination rate and/or germination lag time (Baskin & Baskin 2014). Germination of a given species occurs in a temperature range related to the habitat they live in (*i.e.*, thermic window) and the maturation stage of the seed embryo. Seeds whose germination is regulated by temperature are considered thermoblastic (Orozco-Segovia & Sánchez-Coronado 2013). Furthermore, storage time can affect seed viability; according to their tolerance to dryness, seeds can be classified as recalcitrant if they do not survive drying and/or freezing and germinate immediately after been released, or as orthodox when they can resist drying and/or freezing and remain viable in a seed bank (Hong & Ellis 1996, Bewley *et al.* 2013).

Previous studies in Mexican Podostemaceae have found that (i) light quality, (ii) temperature, (iii) storage time influence their germination (Philbrick & Novelo 1994, Reyes-Ortega *et al.* 2009, Castillo *et al.* 2017). But there is not information for any podostemad species about how seed origin (rivers), and their location, affect seed germination. Recently, Castillo *et al.* (2017) pointed that light incidence at the rivers affect germination rates for both *M. foeniculaceum* and *N. coulteriana*.

Here we studied seed germination response of *N. coulteriana* and *M. foeniculaceum*, two species of Mexican Podostemaceae, collected from four collection sites in Puerto Vallarta, Mexico (Figure 2). Seeds were exposed to different

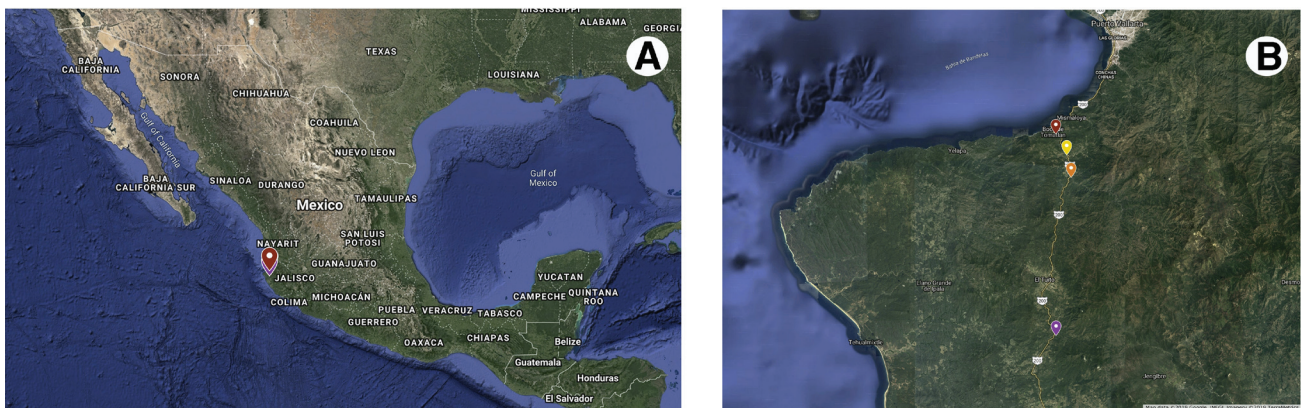


Figure 2. A) Map of Mexican Republic pointing out Jalisco state. B) Approximation indicating the collection sites of fruits of *N. coulteriana* and *M. foeniculaceum* included in this study: ● Horcones River, ● Las Juntas del Tuito River (Las Juntas), ● Las Juntas y Los Veranos river, ● Boca de Tomatlán river.

light qualities (white, red, far-red and darkness) and temperature treatments (constant 25 °C and alternate 15-25 °C). We also explored the influence of storage time (1-17 years) on accumulated seed germination percentage and final seed germination percentage of both species. We hypothesize that seed germination decreases with storage time and that temperature, light quality and collection site affects germination of both studied species.

Materials and methods

Study system. *Marathrum foeniculaceum*. Annual or perennial plant; characterized by repeatedly pinnately divided leaves to 80 cm, hair-like to flatten; roots 0.2-1 mm wide; stems to 20 cm; flowers actinomorphic with pink tepals; capsules 4-6 × 1.7-2.5 mm, seeds 0-1,500 per capsule, 0.24-0.45 × 0.12-0.27 mm; distributed from central Mexico to Colombia (Novelo *et al.* 2009, Garcia-Posada & Muñoz-López 2011, Tippery *et al.* 2011) (Figures 1B-C). *Noveloa coulteriana*. Annual or perennial plant; divided leaves 2-10 cm long; roots prostrated and flattened 15 cm long; capsules 1.6-2.8 × 0.8-1.2 mm, each valve 3-ribbed, 7-73 seeds per capsule, 0.17-0.34 × 0.11-0.25 mm. *N. coulteriana* is a species endemic to Mexico, which is distributed along the Pacific slope from Sonora to Guerrero and in Baja California Sur (Novelo & Philbrick 1997) (Figures 1D-E).

Plant material. Fruits of *M. foeniculaceum* and *N. coulteriana* were collected in non-consecutively yr from 1996 to 2013 (see Appendix 1) in different collection sites of the rivers Horcones and Arroyo del Rincon, both rivers located in Puerto Vallarta, México (Figure 1). Complete and not predated fruits (> 20) of each studied species were collected from randomly selected rocks along the river course at each collection site (Figure 1). Since fruits of both species are naturally dry and dehiscent, fruits were stored in paper bags at room temperature (20±2 °C) until the beginning of germination experiments.

Seed germination experiment. From each collection site and species (Appendix 1), we extracted seeds of 10 randomly chosen fruits to obtain a heterogeneous sample. With a stereoscopic microscope, 30 seeds were sowed in Petri dishes, by triplicate, with absorbent paper and commercial bottle water low in salts to resemble the oligotrophic condition of the rivers. Petri dishes with water was sealed with parafilm and not was open until the end of the experiment in order to maintain humidity constant. Seed germination experiment was carried out during April 2014. Seeds were subjected to a combination of constant (25 °C) and alternate (15/25 °C) temperature with white light (WL), red light (RL) (R: RL = 4.44), far-red light (FRL) (R: RL = 0.05) and darkness (D). For RL and FRL treatments, acrylic Plexiglas boxes with different filters were used to do so, for darkness treatment Petri dishes were wrapped with aluminum foil. All boxes were placed in a controlled environment chamber Biotronette 84 (Labline Instruments, USA) with photoperiod 16/8 day and night, relative humidity at the inside of the chamber

~40 %. Germinated seeds in WL were counted daily with a Zeiss 475262 stereoscopic microscope until germination was depleted. For the other treatments, only the final number of germinated seeds was counted when seed under WL treatment reached asymptote to avoid the interruption of the treatments. We estimated accumulated germination percentage (AGP) for the WL treatment only, and final germination percentage (FGP) for all treatments. The criterion of seed germination was the emergence of the radical pole through the seed coat. We grouped seeds of one and two years (Group I) and seeds of six and seven years (Group II) based on observations made in a preliminary experiment where germination between seeds did not showed significant differences (data not shown).

Statistical Analyses. We used a Cox regression model to study the effect of storage time (Group I vs. Group II), temperature treatments and differences between species upon the time seed germination (AGP) takes to happen in WL treatment. In addition, to study the effect of storage time, light quality and temperature treatments in FGP we performed a quasi-binomial GLM with a logit-link function.

We also explored the effect of light quality, temperature treatments and collection site in seed germination. To do so, we performed a quasi-binomial GLM with a logit-link function with FGP as response variable. To avoid aging effect, we performed this analysis exclusively with data of Group I. Analyses were carried out using R statistical software v. 2.13.0 (R Core Team 2011).

Results

Storage time. Seeds older than 9 yr did not germinate in any case. In WL, seeds from Group I, of both species, began to germinate between days two and three. The highest AGP in *N. coulteriana* was at sixth day at constant temperature and at twelfth day in alternate temperature (Figure 3A); in *M. foeniculaceum* the highest AGP was at day five at constant temperature and by day six for seeds germinated at alternating temperature (Figure 3C). In group II, *N. coulteriana* at constant temperature began germination at day eight and reached the highest AGP at day fourteen, and do not germinate in alternate temperature (Figure 4A); *M. foeniculaceum* started germination between days six and eight at constant and alternate temperature, respectively (Figure 4C), and the highest AGP at day nine in constant temperature, and at day thirteen at alternate temperature. There was a significant effect of the storage time on AGP (see Table 1). The time needed to reach the highest AGP is slower in Group II compared to Group I (Figures 3A, C and 4A, C).

The FGP achieved by both species in Group I in WL was ≥ 90 %. In RL, seeds of *M. foeniculaceum* remained with high FGP ≥ 99% in both temperature treatments, while seeds of *N. coulteriana* decreased significantly ($\chi^2 = 15.1188$, $p = 0.0001$) to about 85 % germination at both temperature treatments compared with WL treatment (Figures 3B, D). In Group II, when seed were exposed to WL we found that the

Germination of two Mexican Podostemaceae for conservation strategies

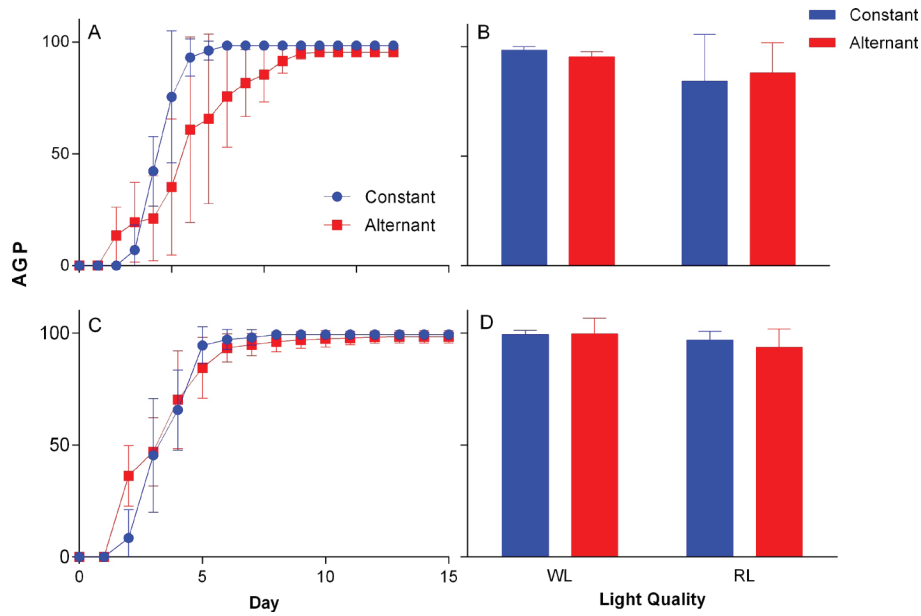


Figure 3. Accumulated germination percentage (AGP) by time intervals and final germination percentage for Group I of *N. coulteriana* (A, B) and *M. foeniculaceum* (C, D), exposed to different temperature and light quality treatments: WL = white light, RL = red light. Error bars represent SD.

FGP for *M. foeniculaceum* was $\geq 96\%$ in both temperature treatment, unlike *N. coulteriana* which FGP reduces significantly ($\chi^2 = 4.4196$, $p = 0.0355$) at constant temperature ($\geq 35\%$) when compared to seeds of Group I and do not

respond at alternate temperature. Under the RL treatment, *M. foeniculaceum* showed high FGP 90-98% while *N. coulteriana* decreased its FGP to 54% with constant temperature, meaning that the effect of alternating temperature reduced

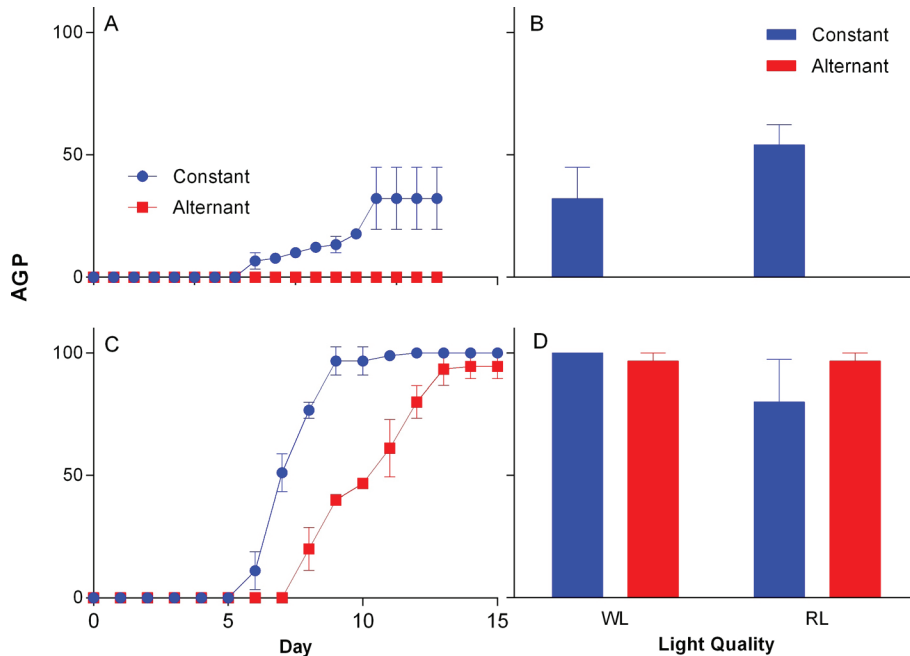


Figure 4. Accumulated germination percentage (AGP) by time intervals and final germination percentage for Group II of *N. coulteriana* (A, B) and *M. foeniculaceum* (C, D), exposed to different temperature and light quality treatments: WL = white light, RL = red light. Error bars represent SD.

Table 1. COX regression that shows the effect of species, temperature treatment and storage time in the accumulated germination percentage of *N. coulteriana* and *M. foeniculaceum*.

Variable	χ^2	<i>p</i>	Risk ratio	95 % lower-upper limits
Species	2.8407	0.0919	1.8366	0.8722-2.9021
Temperature	0.2053	0.6504	2.2731	1.0794-3.5849
Storage time	4.4196	0.0355	1.1432	0.5431-1.8063

significantly ($\chi^2 = 3.9411, p = 0.0471$) germination of Group II of *N. coulteriana* (Figures 4B, D).

Collection site. We found that RL treatment in Group I reduced seed germination of *N. coulteriana* collected from Las Juntas del Tuito, having 71 % of germination compared to the 90 % of the seed germinated under the WL treatment (Figure 5). While seeds of *M. foeniculaceum* from Las Juntas del Tuito, keep germination percentages $\geq 90\%$ in both light treatments (Figure 6). For both species, seeds originated from Horcones river had percentages $\geq 95\%$ in both light treatments. There is a significant difference between seed germination of *N. coulteriana* from different rivers, when applying different light treatments ($\chi^2 = 11.6231, p = 0.0007$). In *M. foeniculaceum* there are not differences that could be attributed to collection site, keeping percentages $\geq 90\%$ in both light treatments. No significant difference was registered in the effect of temperature treatments on the germination capacity for any species or population ($\chi^2 = 2.5327, p = 0.1115$), nor in the interaction with light quality ($\chi^2 = 0.306, p = 0.5801$).

Discussion

As far as we known in Mexico, and all over the world, Podostemaceae seed banking is null. There are only small

collections of seeds belonging to researchers working with the family, and optimal long storage conditions for most of Podostemaceae species are still unknown. It's important to generate information about podostemads seed storage and seed germination for its importance for the species reproduction and population maintenance (Philbrick & Novelo 1995). Here, in agreement with previous investigations, we found that seeds of *N. coulteriana* and *M. foeniculaceum* are positive photoblastic and have no latency (Philbrick & Novelo 1994, Reyes-Ortega 2009, Luna *et al.* 2012, Castillo *et al.* 2013).

Seeds of the Group I showed high germination percentages ($> 90\%$) regardless of temperature and light quality treatments. These levels are interpreted as a strategy that evolved in Podostemaceae in response to the difficulty of finding a suitable microhabitat for seedling establishment in the stressful environments that these species inhabit (Castillo *et al.* 2017). Moreover, storage time affects *N. coulteriana* to a larger extent when compared to *M. foeniculaceum*. Storing reduced germination of both species after 6 yr, however, this effect is more pronounced in *N. coulteriana* than in *M. foeniculaceum*, where storage time only influences the beginning of germination and the time it takes to reach the maximal germination percentage (AGP). This effect is greater that the reported by Reyes-Ortega (2009) who found that seeds of *M. rubrum* and *M. schiedeanum* (now classified as *M.*

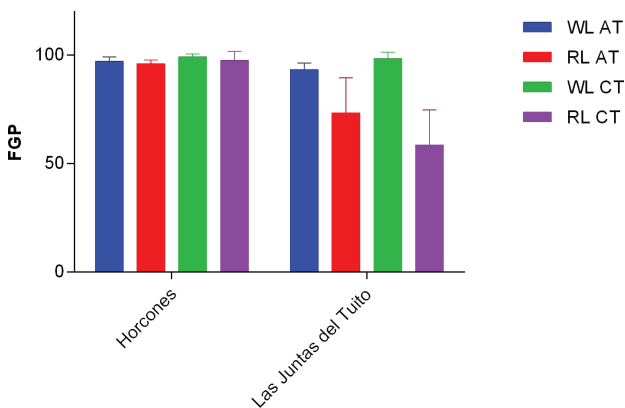


Figure 5. Final germination percentage for Group I of *N. coulteriana* seeds from distinct populations exposed to different temperature treatments (AT = alternant temperature; CT = constant temperature) and the light quality (WL = white light; RL = red light). Error bars represent SD.

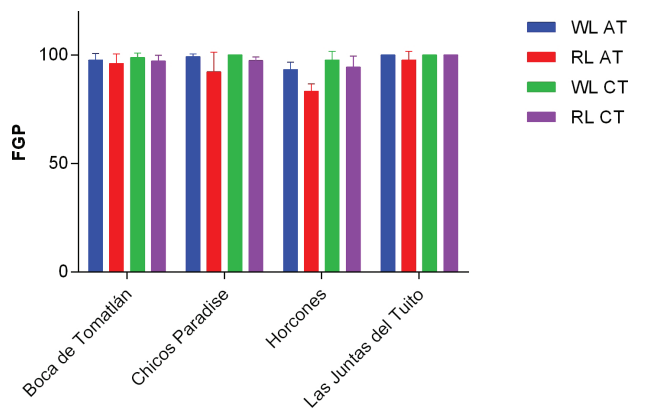


Figure 6. Final percentage of germination for Group I of *M. foeniculaceum* seeds from distinct populations exposed to different temperature treatments (AT = alternant temperature; CT = constant temperature) and the light quality (WL = white light; RL = red light). Error bars represent SD.

foeniculaceum) reduces its FGP to 87 % and 2 % respectively after 3 yr of storage. In the case of *N. coulteriana*, aging decrease FGP ≤ 54 % and even loss germination response in alternant temperature, this decay has also been reported for seeds of six months of storage by Castillo (2013). Delayed speed germination could be attributed to the time needed to repair the structures and restart metabolism due there've been deteriorating for longer (Waterworth *et al.* 2015). Seeds lose its viability when stored ≥ 9 yr.

We also found that *N. coulteriana* is more susceptible to aging, light quality and temperature treatments than *M. foeniculaceum*. WL result to be the optimal for reaching the highest FGP (≥ 90 % and ≤ 35 % respectively) for both species, while RL only decrease significantly FGP in Group I and II (85 % and 54 % respectively) of *N. coulteriana*, reducing FGP to 0 % when alternating temperature is applied to seeds of Group II. Constant temperature is also the optimal to reach high FGP unlike alternating temperature that reduces significantly FGP of *N. coulteriana*, having a significantly effect on those exposed to RL. Results point that light quality have a greater effect on seed germination of *N. coulteriana* seeds than in *M. foeniculaceum*. In this study we discard *N. coulteriana* as recalcitrant (Castillo *et al.* 2013) and propose that seeds of both species are orthodox in agreement with other authors (Philbrick & Novelo 1994, Reyes-Ortega 2009). We consider that the low metabolic activity, the absence of latency and mucilage formed in the seed upon contact with water, are characteristics that together reflect dynamics of seeds in their aquatic environment where germination and seed dispersal will be determined by the water level, which constantly changes over the years. Another feature that allows us to classify those seeds as orthodox is their ability to be stored at very low temperatures (-12 °C) without causing cell damage (Philbrick 1984, Berjak & Pammenter 2010) since it has been seen that may remain stored at very low temperatures without loss of ability for at least 2 months (Philbrick 1984).

There are limited and empirical information in Mexico about pollution of rivers where podostemads grow, some species can tolerate a degree of nutrient loading, but it is not known long-term impacts of these nutrients have on populations (Quiroz *et al.* 1997). Here we analyzed germination response collected from different rivers. We found that *N. coulteriana* population of Las Juntas del Tuito showed a diminution in his FGP under the RL treatment. Although we do not know what the cause of this behavior in the population of Las Juntas del Tuito might be, we suggest that this may be caused by the exposure of the mother plants to the shadow caused by the canopy covering the river at the location, influencing seed germination through maternal effects (Castillo *et al.* 2017, Wulff 2017).

Altogether, our results indicate that *N. coulteriana* is more susceptible to variation in storage conditions (*e.g.*, storage time, light quality and temperature) compared to *M. foeniculaceum*. Moreover, we found that collection site is another factor that can affect seed germination after storing in Podostemaceae. These highlights the importance of testing germination conditions of Podostemaceae at species

level as well as the collection site election in order to find optimal storage options to preserve germplasm. Even though *N. coulteriana* and *M. foeniculaceum* can potentially form *ex situ* seed banks up to 8 yr of age, seeds older than 9 yr did not germinate in any case.

These results point that long-term conventional *ex situ* seed germination storage is not a viable strategy to conserve germplasm of Podostemaceae. *Ex situ* plant conservation depends on the species, the methods employed and the desired storage time (Li & Pritchard 2009), so further studies should explore different mechanisms of maintenance as seed desiccation tolerance and storage at sub-zero conditions. Such studies need to explore the viability of seed banking using cryopreservation techniques that can help us to protect Podostemaceae from habitat loss and potential species extinction. Besides knowing the optimal storage conditions and time for seeds of podostemads, it will be also very important to know the capacity of the plants to establish in their environment after being stored for several years in *ex situ* seed banks.

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Appendix 1. Collects of *N. coulteriana* and *M. foeniculaceum* from different locations and seasons since 1996 to 2013.

Species	River	Location	Collect season	Time of storage (years) until the moment of germination	
<i>N. coulteriana</i>	Horcones	Los Horcones 20° 27' 9.39" N 105° 17' 22.99" W	1996-1997	17	
			2007-2008	6	
			2011-2012	2	
			2012-2013	1	
	Arroyo del Rincón	Las Juntas del Tuito 20° 14' 47.25" N 105° 18' 38.04" W	2011-2012	2	
<i>M. foeniculaceum</i>	Horcones	Horcones	2004-2005	9	
			2012-2013	1	
			Las Juntas y los Veranos 20° 28' 54.33" N 105° 17' 44.98" W	2012-2013	1
				2011-2012	2
			Boca de Tomatlán 20° 30' 32.52" N 105° 18' 39.68" W	2012-2013	1
	Arroyo del Rincón	Las Juntas del Tuito	2004-2005	9	
			2006-2007	7	
			2011-2012	2	