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Substrate, moisture, temperature and seed germination of the threatened endemic tree *Eriotheca vargasii* (Malvaceae)

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Abstract: We studied the germination of *Eriotheca vargasii* (Malvaceae), a poorly known endemic Peruvian Andean tree species characteristic of the dry forests of the Torobamba river valley, Peru. We determined seed characteristics, embryo morphology, viability, and assessed the influence of substrate (natural soil and commercially prepared media), temperature (controlled at 25 ºC and at ambient temperature between 18-22 ºC), and moisture (25 % and 50 % field capacity) on seed germination. Most seeds were ovoid in shape and although they contained well-developed embryos, only 46 % of them were viable. Substrate moisture levels had no influence on germination capacity or rate. In contrast, temperature and substrate type showed strong effects on germination. We observed the highest proportion of germinated seeds in prepared media at both temperatures tested (> 61 %). Furthermore, substrate types also influenced germination rates, with lower values in natural soil. The strongest effect on germination rates was by temperature, enhancing the difference in responses in substrate types (up to 90 % in commercially prepared media at 25 ºC). The low proportion of germinated seeds in soil (< 39 %), together with external local stress factors (e.g. grazing impact by herbivores), may be the critical factors contributing to the nearly total absence of seedlings and saplings of *E. vargasii* in the study area despite abundant seed production. In order to ensure a supply of *E. vargasii* seedlings for reforestation efforts, we recommend producing *E. vargasii* plants in nurseries and conducting reforestation trials. We suggest that germination of seedlings is done following guidelines from this study. Rev. Biol. Trop. 66(3): 1162-1170. Epub 2018 September 01.

Key words: Andes; arid ecosystem; drought; seed morphology.

Seedlings growing in the seasonally dry tropics face challenging conditions such as seasonal water deficits, high insolation, high temperatures and transpiration rates (Harms & Paine, 2003; García-Núñez, & Azócar, 2004). Woody species growing in these habitats have thus developed several strategies to overcome such adverse conditions. For example, seedlings from most woody species from seasonally dry vegetation in Bolivia, Brazil and Venezuela are capable of rapidly accumulating root biomass in the form of a strong taproot system or of water storage organs during their first rainy season to survive the following dry season (Moreira & Klink, 2000; García-Núñez & Azócar, 2004; Poorter & Markesteijn, 2008). Some species begin seed germination as soon as they are dispersed, others maintain seeds viable in the seed bank and await more humid and favourable conditions (Dalling, 2002; Ceccon, Huante, & Rincón, 2006; Vieira, Lima, Sevilha, & Scariot, 2008), while some have intermittent germination (Murdoch & Ellis, 2000).

The South American genus *Eriotheca* (Malvaceae-Bombacoideae) includes 24 species, most of which are characteristic trees of

seasonally dry tropical forests (SDTF) (Duarte, 2010). Members of this genus present a dehiscent ligneous capsule that opens at maturity into five valves. The fruit contains a densely hairy whitish to reddish endocarp (kapok) with numerous glabrous seeds (Duarte, 2010). The fruiting season for *E. ruizii* in the Northern coastal SDTF has been reported between July and December (Martos, Scarpati, Rojas, & Delgado, 2008). A similar phenology has been noted for *Eriotheca vargasii* (R. Linares-Palomino, pers. obs.) a poorly known Andean tree, endemic to the dry valley system of the Apurímac river and its tributaries in southern Andean Peru (Reynel, Pennington, Pennington, Marcelo, & Daza, 2003; León, 2006). In a recent survey to characterize the adult population of *E. vargasii* in a remnant dry forest patch in Ayacucho (inter-Andean Southern Peru), Linares-Palomino (2013) did not find signs of regeneration, nor a single juvenile individual. Potential factors contributing to the lack of regeneration included the presence of livestock (mainly goats), steep and fragile terrain, and intrinsic factors like pollination failure, insufficient seed and fruit production, lack of seed dispersal and poor seedling establishment, or a combination thereof (Linares-Palomino, 2013). There is little information about the ecology of members of this genus, less so about their seed and germination ecology, and all available knowledge comes from two Brazilian savanna and rain forest species, respectively, *E. gracilipes* and *E. pentaphylla* (Fischer, 1997; Mercival, Lunardi, Guimarães, & Galetti, 2008; Melo, Cavalcanti, Alves, Martins, & de Araújo, 2017). Non-dormant seeds and physical dormancy due to a water-impermeable layer in the seed coat have both been reported for the Malvaceae (Baskin & Baskin, 2001). The latter seems to be a response of dry forest species to the dry season and rainfall variability (Baskin & Baskin, 2001), but we are not aware of any specific study reporting it for dry forest species of *Eriotheca*. While additional information on seed morphology has also been generated for *E. ruizii* (Alvarado & Gonzalez, 2009), there is a dearth of information for all *Eriotheca* species, and in particular for those in the isolated inter-Andean valleys.

There is clearly a need for better knowledge on seed germination and seedling establishment in SDTF in general, not only as a means to understand key forest community processes, but also to produce information for conservation and restoration efforts (Khurana & Singh, 2001). SDTF are considered the most threatened forest ecosystem globally, facing intense fragmentation and land-conversion and making them a region-wide conservation priority (Banda et al., 2016). Therefore, with the aims of 1) identifying if the seed germination process in *E. vargasii* could be a factor to be considered in explaining the observed population structure of this tree species as reported above, 2) contributing to the seed ecology of *E. vargasii*, and 3) providing recommendations for successful *E. vargasii* seed germination, we present here a study assessing its seed characteristics, seed viability, and optimal conditions for germination in relation to substrate, ambient temperature and moisture.

MATERIALS AND METHODS

Seed source and selection: We collected dry mature fruits containing kapok and seeds from at least 40 different *E. vargasii* trees from the SDTF in the inter-Andean valley of the Torobamba River (Ayacucho, 2 300 masl) during the dry season of August 2012, when *Eriotheca* fruits are available (Fig. 1). These valleys are characterized by hot (20-24 °C) and arid (200-1 000 mm annual rainfall) conditions. The study area receives 536 mm of annual rainfall (2 612 masl, San Miguel, Ayacucho), with 352 mm concentrated in the rainy season (December to March) (Servicio Nacional de Meteorología e Hidrología, 2012). Dry dehiscent fruits were collected directly from the trees, placed in paper bags and maintained at ambient temperature. We separated the seeds from the kapok and selected 1 700 seeds that (i) had good physical condition (excluding shrivelled, damaged seeds) and (ii) passed a flotation test

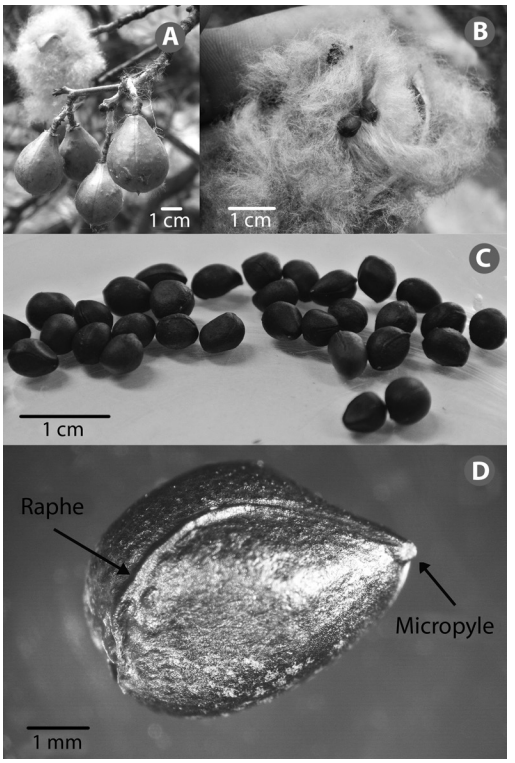

Fig. 1. External morphological fruit and seed characteristics of *Eriotheca vargasii*. **A.** ovoid fruits, **B.** dehiscent fruit with seeds embedded in kapok, **C.** black coloured seeds, **D.** external seed morphology.

in water (floating seeds were considered nonviable; Merritt, 2006).

Seed characterization: We used dry seeds to record length, width, thickness, weight, shape and colour (Munsell colour chart) from 50 randomly selected seeds. We used the Tetrazolium method to quantify viability of additional 100 randomly selected seeds. We first removed the testa and put all seeds in a 1 % Tetrazolium salt solution for four hours at 30 ºC (International Seed Testing Association, 2012). We then washed all seeds with water and removed the remaining seminal covers to facilitate observing colouring (or lack thereof) of the embryo (Delouche, Still, Rapset, & Lienhard, 1962).

We used an ALC-210.4 analytical balance (ACCULAB North America, CO, USA) for weight measurements and a Leica LED2500 stereoscopic microscope (Leica Microsystems, Switzerland) for recording observations.

Germination experiment: We randomly selected 1 200 additional seeds to assess the influence of three factors: substrate (soil versus vermiculite), temperature (25 °C constant versus ambient between 18-22 °C) and moisture (humidity at 25 % versus 50 %), on germination capacity (the total number of seeds germinated, expressed in percentages) and germination rate (number of seeds germinated per day, expressed in percentages) (Ranal & Santana, 2006). This resulted in eight different treatment combinations, with three replicates for each treatment (of 50 seeds each).

To test for substrate, we used natural and commercially prepared media. Parallel to the seed harvesting activities, we also collected soil from the upper 15 cm of one square meter area in the Torobamba SDTF. According to the manufacturer, the Sunshine Premix 8 (Sun Gro Horticulture Inc., MA) contained *Spagnum* peat moss, vermiculite and perlite, dolomite, gypsum and proprietary starter nutrient charge with major and minor nutrients as well as proprietary wetting agents. The substrates were placed in transparent plastic boxes under an 8 hr light / 16 hr dark photoperiod. We performed tests with two temperature conditions. First, under controlled conditions at 25 ºC of constant room temperature (INIA, Lima), and second, under ambient laboratory temperature varying between 18-22 °C (INIA, Huamanga). We also tested germination at 50 % and 25 % of substrate moisture at field capacity. The former treatment consisted in maintaining seed adequate moisture but allowing ventilation, adding 187 ml of water per 100 g of Premix substrate and 18 ml of water per 100 g of soil to attain the desired moisture content. The latter field capacity consisted in maintaining moisture content at minimum by adding 94 ml of water per 100 g of Premix substrate 9 ml of water per 100 g of soil.

During 21 days we recorded the number of germinated seeds in each treatment, as well

as recording death events. Germination was assessed visually, and a seed was considered germinated when the hypocotyl had emerged. Finally, we calculated daily cumulative germination and germination capacity (Ranal & Santana, 2006). After the experiment, we evaluated all remaining seeds in each treatment to check for non-viable seeds.

Data analyses: Differences in germination capacity were assessed with contingency tables through a G-test for proportions of germinated seeds for each treatment. To test whether temperature, substrate or moisture levels influenced germination rate in *Eriotheca vargasii*, we performed a survival analysis. This allowed us to account for temporal autocorrelation in seed germination records. We evaluated Cox proportional hazard models including temperature, substrate and moisture as predictors, and germination (considering censored events) as response. Subsequent model validation was performed to assess proportional hazards (PH) assumption with posterior addition of stratification or interactions terms (Kleinbaum & Klein, 2012; McNair, Sunkara, & Frobish, 2013). Stratification was applied over the variable that did not satisfy the PH assumption, generating different baseline hazard functions at each level of the variable. In addition, interaction between predictors allowed us to estimate separate regression coefficients for each stratum in case the non-interaction model did not satisfy the PH assumption (Kleinbaum & Klein, 2012). For G-test and Cox PH model analyses we performed tests for combined data of replicates or variables after detection of non-significant differences in germination capacity or germination rate between them. The final sample size for each treatment included only the germinated seeds and those considered without visible damage or fungal infection at the end of the experiment (Table S1). All analyses were performed with R (R Core Team, 2016) using packages "survival" (Therneau, 2015) and "DescTools" (Signorell, 2016).

RESULTS

Seed characteristics: We identified three main colours, varying between black (48 %, Fig. 1C), red (34 %), grey (10 %) and combinations thereof. The testa of *Eriotheca vargasii* had a rugulose, scarcely wrinkled surface. The micropyle was visible (Fig. 1D). We found that most seeds were ovoid in shape (90 %), but also recorded spherical (4 %) and elliptic (6 %) seeds. Seeds had an average length of 5.58 mm (±0.05 mm), width of 4.09 (±0.03 mm), thickness of 4.08 (±0.03 mm) and weight of 0.03 g (±0.01 g). We found no significant ($P > 0.2$) correlation between weight and any of these morphological variables. We found that 46 % of the seeds were viable using the Tetrazolium test. Within the non-viable seeds, we observed seeds with fungal infection, empty seeds (i.e. without a developed embryo) and seeds with damaged embryos.

Germination: Germination capacity did not vary between replicates for each combination of treatments (G-test, all pairwise comparisons, $P > 0.05$). The proportion of germinated seeds for the combined data did not show differences between levels of moisture, for any level of temperature or substrate compared (G-test, all pairwise comparisons, $P > 0.05$). In contrast, germination capacity was lower in soil at 25 °C (16 %, G: 183.81, *d.f.* = 1, $P < 0.01$) than at 18-22 °C (39 %, G: 19.14, *d.f.* = 1, $P < 0.01$), while the highest proportions of germinated seeds were found in premix substrate at 25 °C (90 %) and at 18-22 °C (61 %), with proportions being significantly different (G: 39.1, *d.f.* = 1, $P < 0.01$, Fig. 2).

Germination rate did not differ between replicates across all combinations of temperature, substrate or moisture (log-rank test, all pairwise comparisons, $P > 0.05$). Substrate humidity did not affect germination rate at any level of temperature or substrate type (likelihood ratio test, $\chi^2 = 0.68$, *d.f.* = 1, $P = 0.41$, Fig. 3). However, both temperature and substrate type had strong effects on germination rates. We detected significant differences between

substrate types (proportional hazards, $\chi^2_{substrate}$ = 169.12, *d.f.* = 1, P < 0.001), with lower seed germination rates in soil substrates (Fig. 4). Temperature had an even stronger effect on germination, since proportional hazards were not constant between 25 °C and 18-22 °C. Indeed, we found that temperature determined a different baseline survival function at each level (likelihood ratio test, $\chi^2_{strata\ (temperature)}$ = 41.57, *d.f.* = 1, P < 0.0001, Table S2). In addition, differences in germination curves in soil or premix substrates were influenced by temperature (likelihood ratio test, $\chi^2_{substrate\ x\ temperature}$ = 41.58, *d.f.* = 1, P < 0.0001, Table S2),

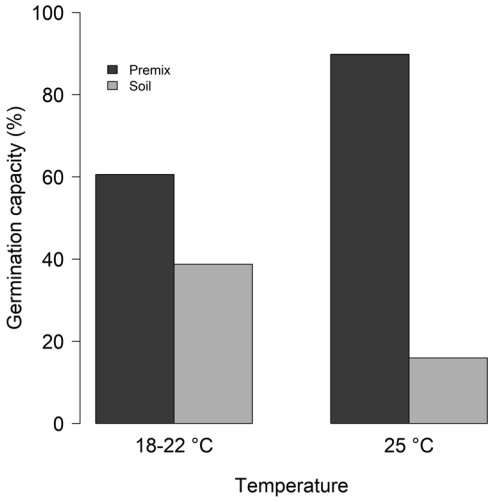

Fig. 2. Germination capacity of *Eriotheca vargasii*, for combined data at each temperature and soil treatment (18-22 °C: n_{premix} = 208, n_{soil} = 191, 25 °C: n_{premix} = 138, n_{soil} = 163), with the highest proportion in premix substrate at both temperatures.

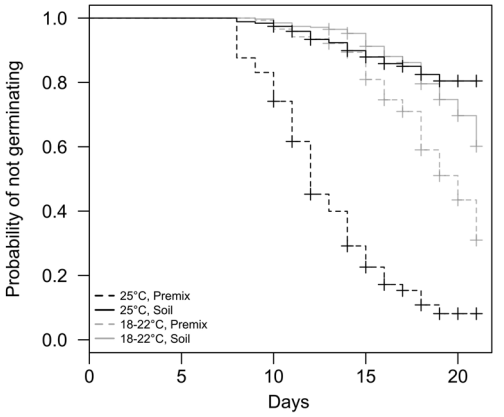

Fig. 4. Optimal Cox proportional hazard model of germination rate in *E. vargasii* stratified by temperature (different baseline, non-proportional hazard functions), and contrasted by type of substrate.

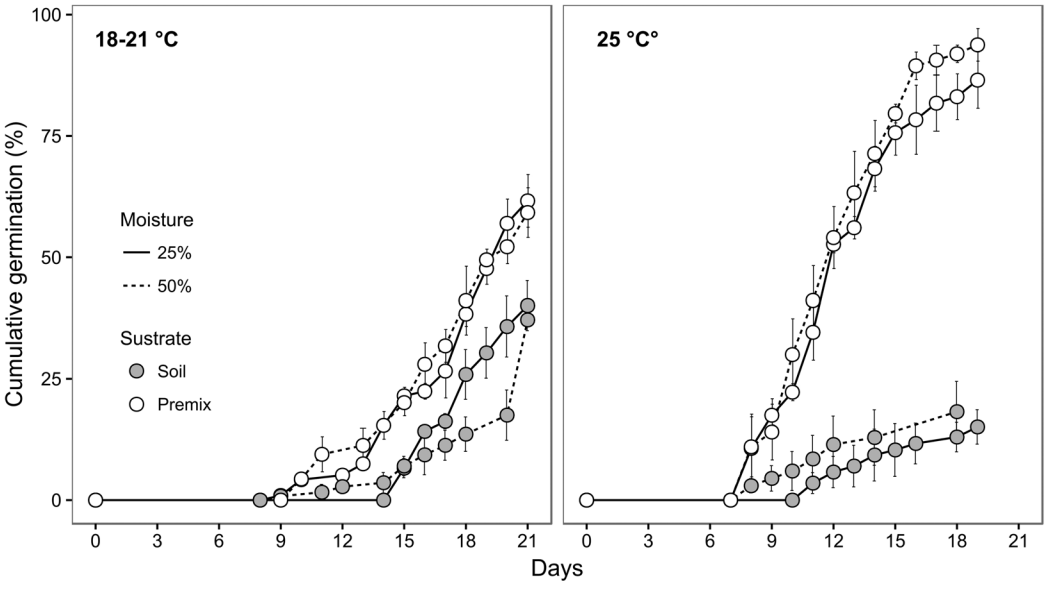

Fig. 3. Cumulative germination of *E. vargasii* along 21 days of experimentation. Notches represents standard error (± SE) of cumulative germination between three replicates each day.

where the effect of substrate is larger at 25 °C than 18-22 °C (Fig. 3).

DISCUSSION

Our results from the morphological analyses agreed well with those reported by Alvarado & Gonzalez (2009) for *Eriotheca ruizii*, a SDTF tree from the Equatorial Pacific forests in south-western Ecuador and north-western Peru. Seed dimensions and the prevalence of ovoid shape were similar in both studies. In contrast to *E. vargasii*, *E. ruizii* seeds were uniform in coloration, usually black with creamish patches. Embryo characteristics were also similar to *E. ruizii* with a solid, globose, cream-whitish, glabrous embryo, with fused cotyledons forming a single structure with the rest of the embryo.

Our results fall within the recorded range of published studies on germination rates for species in the bombacoid subfamily. Román, De Sautu, Deago, Hall, & Jefferson (2012) reported germination rates between 38 % and 86 % and for five species (*Ceiba pentandra*, *Ochroma pyramidale*, *Pachira quinata*, *Pseudobombax septenatum*, *Quararibea asterolepis*) growing in both dry and humid forest in Panama. Fischer (1997) performed germination essays on three bombacoid species (*E. pentaphylla*, *E. candolleana*, and *Ceiba speciosa*) attaining germination rates between 33 and 83 % (79-83 % for *Eriotheca* species). Germination rates for four bombacoid species present in forests in Quintana Roo (Mexico), showed values between 78 % and 88 % (Sánchez Sánchez & Hernández Zepeda, 2004). Carrijo et al. (2009) explored the utility of *E. pubescens* for the restoration of degraded forests in Brazil. Laboratory essays showed that vermiculite was the most adequate substrate with mean germination rates of 77.5 %, whereas cotton and filter paper attained 58 % and 45 %, respectively. Field essays, where seeds where directly sowed into a prepared (tillaged) but abandoned mining area, showed germination rates of nearly 80%, suggesting an opportunity of restoration success in the Brazilian Cerrado.

The marked deciduousness of SDTF species, make the dry season the best time for seed dispersal of anemochorous species (Luz & Nunes, 2013) such as *E. vargasii*. However, environmental conditions during the dry season (April-November) experienced by plant species in the inter-Andean SDTF of our study area, which include high diurnal temperatures, limited water availability and exposure to high solar radiation (R. Linares-Palomino, unpub. data), make it an unsuitable time for germination, as has been shown by studies in similar environments across the dry tropics (Khurana & Singh, 2001; Ceccon et al., 2006). *Eriotheca vargasii* seeds have a hard testa, a characteristic suggested to aid survival under harsh conditions (Moreno, 1996) by delaying germination until the rainy season (Vieira et al., 2008). Seed dispersal of *E. vargasii* has been observed to peak during July and August each year (R. Linares-Palomino, pers. obs.), but germination delay would avoid seedling desiccation and thus intermittent recruitment (Khurana & Singh, 2001).

SDTF regeneration trials suggest that woody species do not form a persistent seed bank (Alvarez-Aquino, Barradas-Sánchez, Ponce-González, & Williams-Linera, 2014), germinating after seed dispersal with the onset of the rainy season (Salazar, Goldstein, Franco, & Miralles-Wilhelm, 2011). Once favourable conditions are met, it is important for seeds to rapidly germinate and develop the seedling in order to take maximum advantage of the short rainy season (Mostacedo & Fredericksen, 2001). The low germination percentages we observed in soil under controlled conditions (as compared to other bombacoid species) suggest lower values under field conditions. This may be one factor contributing to the nearly total absence of seedlings and saplings of *E. vargasii* in the study area immediately after the rainy season, despite abundant seed production (Linares-Palomino, 2013). An additional factor likely influencing these low seedling densities reported is the presence of goats and, to a lesser extent, cattle in the study area. Current livestock exclusion experiments underway in

our study area will help disentangle the effect of intrinsic germination-related factors and browsing pressure due to livestock.

In order to fill these existing knowledge gaps and generate the information necessary to manage and conserve the SDTF biome we need to identify other plant species under similar threatened conditions as the ones described here for *E. vargassi*. We also need to encourage further seed characterization and ecological research for SDTF species. For *E. vargassi* in particular, future research needs to test if the seed coat color variations that we have observed has any influence on seed germination. We also need to look at how viability, dormancy and patterns of germination change after seeds are dispersed and until the onset of rains. Some tree species subjected to monsoon climate in India, for instance, reduced seed viability immediately after dispersal (Murali, 1997), increasing again towards the rainy season. Other dry forest tree species showed best germination results at the end of the dry season or early in the wet season (Fleming, Williams, Bonaccorso, & Herbst, 1985; Flores & Rivera, 1985). Several factors responsible for breaking this physical dormancy have been described (Baskin & Baskin, 2001). As suggested by our results, the environmental changes triggering germination of *E. vargasii* in the inter-Andean SDTF of our study site seem to be related to ambient water availability and temperature thresholds (Baskin, Baskin, & Li, 2000; Baskin, 2003; Soriano, Huante, Gamboa-deBuen, & Orozco-Segovia, 2014).

We also need to test pre-treatment methods that can help us improve germination through e.g. physical or chemical scarification, contrast germination under field conditions with our present laboratory-based knowledge and look into mycorrhizal interactions with SDTF plant species (Khurana & Singh 2001). This study showed that the premix substrate under controlled temperature conditions of 25 °C are recommended for germination trials of this species. This is only a first step towards understanding and protecting this species and ecosystem. The implementation of additional studies following the lines of research stated above and the development of activities in collaboration with the local communities can help recover and conserve these fragmented and disturbed SDTFs, as has been successfully shown in Ecuador through intensive tree planting of selected native species (Horstman, Ayón, & Griscom, 2017).

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RESUMEN

Sustrato, humedad, temperatura y germinación de semillas del árbol endémico amenazado ***Eriotheca vargasii*** **(Malvaceae).** Estudiamos la germinación de *Eriotheca vargasii* (Malvaceae), un árbol endémico poco conocido de los andes peruanos y carácterístico de los bosques secos del valle del río Torobamba. Determinamos las características de la semilla, la morfología del embrión y la viabilidad; además evaluamos la influencia del sustrato (suelo y sustrato preparado comercialmente), temperatura (controlada a 25 ºC y sin control entre 18-22 ºC) y humedad (25 % y 50 % de capacidad de campo) sobre la germinación de las semillas. La mayoría de las semillas tuvo forma ovoide y aunque la mayoría contenía embriones bien desarrollados, sólo el 46 % de estas fue viable. Los niveles de humedad del sustrato no tuvieron influencia sobre la capacidad o tasa de germinación, mientras que la temperatura y el tipo de sustrato si tuvieron efectos visibles. Observamos las proporciones más altas de semillas germinadas en los sustratos preparados y en ambas temperaturas (> 61 %).

Adicionalmente, el tipo de sustrato también influenció las tasas de germinación, con valores más bajos en el suelo natural. El efecto más fuerte sobre las tasas de germinación se dió por la temperatura, aumentando las diferencias de las respuestas de acuerdo al tipo de sustrato (hasta un 90 % de germinación en sustratos preparados comercialmente y a 25 ºC). La baja proporción de semillas germinadas en el suelo (< 39 %), junto con factores locales de estrés externos (como por ejemplo el impacto por herbivoría), pueden ser elementos clave que están contribuyendo a la casi ausencia total de plántulas de *E. vargasii* en el área de estudio, a pesar de una producción de semilla abundante. Para asegurar proveer plantones de *E. Vargasii* para esfuerzos de reforestación, recomendamos producir plantas de esta especie en viveros y realizar experimentos de reforestación. Sugerimos que la germinación de las plántulas se haga siguiendo las recomendaciones de este estudio.

Palabras clave: Andes; ecosistema árido; sequía; morfología de semilla.

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SUPPLEMENTARY MATERIALS

TABLE S1
Sample size for each experimental treatment considering only viable seeds. Non-viable seeds were determined by phytosanitary evaluation after the experiment

Temperature	Substrate	Moisture	Replicate	Viable seeds			Non-viable seeds
				Dead	Germinated	Not germinated	
18-22 °C	Soil	25 %	A	11	13	6	20
			B	16	9	5	20
			C	15	15	2	18
		50 %	A	20	16	5	9
			B	14	11	4	21
			C	13	10	6	21
	Premix	25 %	A	16	19	0	15
			B	4	14	6	26
			C	5	26	5	14
		50 %	A	5	27	7	11
			B	12	21	7	10
			C	9	19	6	16
25 °C	Soil	25 %	A	9	6	17	18
			B	4	5	18	23
			C	5	2	18	25
		50 %	A	11	5	6	28
			B	16	6	1	27
			C	32	2	0	16
	Premix	25 %	A	0	24	1	25
			B	5	19	1	25
			C	2	14	0	34
		50 %	A	0	23	3	24
			B	2	26	0	22
			C	0	18	0	32

Total words: 5652

Table S2. Cox proportional model comparison of germination in *Eriotheca vargasii.* The optimal model included substrate type and temperature, where the latter also determined different baseline functions at 25 °C and 18-22 °C. (*** denotes highly significant effects)

Cox model	*logL*	Δ*logL*	χ^2	*d.f.*	*P*
$h(t, \text{Germ}) \sim \beta_{1\,(\text{Sustrate})} + \beta_{2\,(\text{Temperature})} + \beta_{3\,(\text{Moisture})}$	-2048.6				
$h(t, \text{Germ}) \sim \beta_{1\,(\text{Sustrate})} + \beta_{2\,(\text{Temperature})}$	-2049	-0.4	0.68	1	0.41
$h(t, \text{Germ}) \sim \beta_{1\,(\text{Sustrate})} + strata_{\,(\text{Temperature})}$	-1845.1	203.9	407.71	1	$2.2e^{-16}$ ***
$h(t, \text{Germ}) \sim \beta_{1\,(\text{Sustrate})} + strata_{\,(\text{Temperature})} + \beta_{2\,(\text{Sustrate } x \text{ Temperature})}$	-1824.3	20.8	41.57	1	$1.14e^{-10}$ ***