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INITIAL SEED WEIGHT AND SCARIFICATION AFFECT *in vitro* GERMINATION OF *Echinocactus parryi* (Engelm.)

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ABSTRACT

The endemic and threatened species *Echinocactus parryi*, native to the municipality of Juarez, Chihuahua, Mexico, presents important limitations for its reproduction and natural propagation. It is hypothesized that initial seed weight and scarification would affect the percentage of seed germination of this species. The objective was to compare the effect of initial seed weight (two levels) and scarification treatments (four levels) on *in vitro* germination of *E. parryi.* The morphology of seedlings grown for 12 months was also described. Four hundred seeds were divided into two groups of 200 seeds each: first group, seeds of 35 to 45 mg; and second group, seeds of 46 to 55 mg. The scarification treatments were a control (intact seeds), mechanical scarification, application of 25 % sulfuric acid and Murashige and Skoog growing medium added with 2 mg L^{-1} of gibberellic acid. Fifty seeds per treatment were placed for each weight group. Treatments were distributed in a completely randomized experiment with factorial arrangement. Data were subjected to analysis of variance and means were compared by Tukey's test ($p \geq 0.05$). Results showed significance in the interaction of seed weight and mechanical scarification treatment by obtaining 93.2±3.6 % germination in seeds of 35 to 45 mg and 96.0±3.3 % in seeds of 46 to 55 mg at 30 d of *in vitro* culture. The morphological description of the seedlings provided information on the development of *E. parryi* in juvenile stages, which differs from the adult stage. Results on germination and the morphological description of the seedlings represent an important advance for propagation and conservation purposes. To our knowledge, this is the first report on scarification treatment in seeds of different weights and the influence on germination, that also recorded the seedling morphology of *E. parryi.*

Keywords: Cactaceae, endangered species, endemic species, mechanical scarification.

INTRODUCTION

The species *Echinocactus parryi* belongs to the family Cactaceae, is endemic to the municipality of Juárez, Chihuahua, Mexico, and is listed in NOM-059-SEMARNAT-2010 as a threatened species. The main problems it faces are illegal looting, the opening of agricultural and livestock activities, urban growth, low percentages of seed viability, long germination periods and slow growth (García-González *et al.*, 2020).

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Cactus seeds usually have different degrees of dormancy and germinate after a long period of time. Under natural conditions the main factors influencing germination of these species are water, temperature, and light, with lack of moisture being the most limiting factor (Bauk *et al*., 2016). Various alternatives have been used to increase germination rates and percentages. One of them is the mechanical scarification which includes manual sanding, incisions in the seed coat (*testa*), washing or soaking. With mechanical scarification, natural friction conditions are simulated, and the hard cover is modified to facilitate germination (Rodriguez *et al*., 2019). In chemical scarification sulfuric acid is applied, which is a corrosive compound, and simulates the passage of seeds through the digestive tract of animals, increasing the permeability of the coating and accelerates germination (Koi *et al*., 2016). The addition of growth regulators such as gibberellic acid (AG_3) to the growing medium promotes germination and reduces seed dormancy. AG_3 controls physiological properties such as the activation of enzymes that hydrolyse the endosperm, which is partially responsible for the mechanical resistance that delays germination. AG_3 also promotes embryo growth which is suppressed by abscisic acid present in the embryo (Savaedi *et al.*, 2019).

Seed size and weight are factors that determine germination success, seedling development and survival. The greater the seed mass, the more vigorous the seedlings produced (Ruíz-Pérez *et al*., 2021). Other important factors in germination are age, maturity stage and seed genotype (Amador-Alferez *et al*., 2013).

The arid or semi-arid natural conditions in which cactaceae plants develop determine the morphological characteristics such as plant shape, stem height and diameter, and the appearance of the ribs, spines and trichomes. The study of these characteristics is valuable for the morphological interpretation of these species, and it is accepted that plant taxonomy should include both juvenile and adult stages (Loza-Cornejo and Terrazas, 2011).

The objective of this study was to compare the effect of four scarification treatments and the influence of seed weight on *in vitro* germination of *Echinocactus parryi;* assembling the morphological description of seedlings germinated *in vitro* for 12 months. This is the first report on the scarification treatment of seeds of different weights and their influence on germination, as well as the morphology of *E. parryi* seedlings.

MATERIALS AND METHODS

Number of seeds and fruit size

Ripe fruits of *E. parryi* were collected in a natural population, located south of the municipality of Juarez, Chihuahua, Mexico, in the months of July-August 2018. Fruits were transferred to the Plant Biotechnology Laboratory located at the Institute of Biomedical Sciences at the Autonomous University of Ciudad Juarez.

Six fruits belonging to three plants were sampled and collected (Figure 1A). These were ripe, soft in consistency, green, containing black seeds at the time of dissection. Fruits dissected with yellow seeds inside were considered unripe (Figure 1B). The longitudinal size and diameter of ripe fruit were measured with a digital vernier

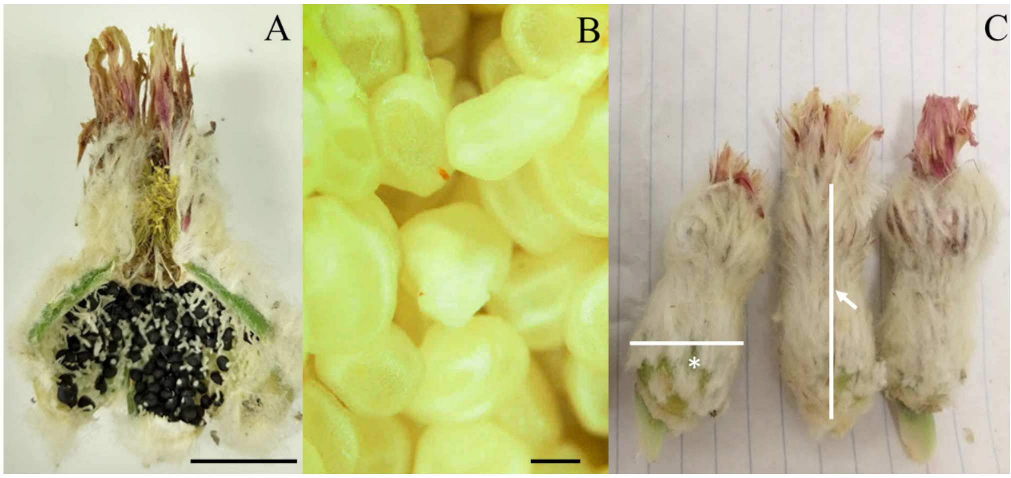

Figure 1. Seeds and fruits of *Echinocactus parryi.* A: fruit dissected longitudinally, showing seeds in black clusters just at the base of the fruit (bar represents 2 cm); B: unripe yellow seeds (bar represents 500 μm); C: morphology of physiologically ripe fruits. The drawn lines above the fruits show the length (arrow) and width (asterisk).

(General®; Figure 1C). Subsequently, each fruit was dissected longitudinally with a scalpel and the seeds were removed with pincers. Fruit size, maximum and minimum number of seeds were evaluated. Seeds were placed in manila paper envelopes and stored in dark, dry conditions at an average temperature of 26 °C for one year.

Influence of scarification treatment and seed weight on germination.

Ripe seeds were weighed on an analytical balance (Sartorius®) and divided into two groups according to weight. Group 1 included seeds from 35 to 45 mg and group 2 from 46 to 55 mg. Both groups of seeds were subjected to four scarification treatments to compare their germination percentage. The first treatment was the control with intact seeds; the second was mechanical scarification, which included the removal of 50 % of the testa using pincers and scalpel (García-González *et al.*, 2020). The third treatment was to sow the aseptic seeds in Murashige and Skoog (MS) culture medium added with 2 mg L^{-1} of gibberellic acid (AG_3) reagent grade (Casisson Laboratories®). In the fourth treatment, seeds were immersed in 25 % sulfuric acid (H_2SO_4) for 15 min under constant agitation and rinsed three times with sterile distilled water. Aseptic seeds were sown on MS medium (at ¼ of their original concentration) and added with 30 g L^{-1} sucrose. The pH of the culture medium was adjusted to 5.7 ± 0.1 with 1N KOH and solidified with 7 g L^{-1} agar (type I for micropropagation; Caisson Laboratories®). The culture medium was autoclaved (Terlab®) at 121 °C for 20 min, and then poured into 125 mL Gerber® flasks containing 25 mL of the culture medium. A total of 400 seeds were used, 200 in each weight group. For each treatment, 50 seeds were used with 10 seeds per jar. The flasks were kept in a growth chamber with a photoperiod of 16 h light with fluorescent lamps with a luminous intensity of 111 μmol m^{-2} s^{-2} at a temperature of 25 ± 1 °C. The criterion for considering a seed as germinated was the emergence of the radicle. Seed germination was monitored every 48 h for 30 days. The effect of scarification treatment, seed weight, and the interaction between both factors on the percentage of *in vitro* seed germination were evaluated for 30 days.

Morphology of *in vitro* germinated seedlings

For the morphological description, seedlings obtained from *in vitro* germinated and mechanically scarified seeds were used. Aseptic seedlings were removed from the flask and placed in a glass Petri dish for observation under a stereoscope (Nikon SMZ 800) and photography at different ages (Nikon Digital Sight DS-Fi2 digital camera). A total of eight seedlings were evaluated.

Statistical analysis

In order to evaluate treatments, a completely randomized experiment with factorial arrangement was used; the study factors were scarification treatments (four levels: control, scarified, AG_3 2 mg L^{-1} and 25% sulfuric acid), and seed weights (two levels: group 1 with seeds of 35 to 45 mg, and group 2 with seeds of 46 to 55 mg). Analysis of variance was performed, and means were compared by Tukey's test ($p \leq 0.05$) using SPSS version 25.0. Due to data variation over a range of 0 to 100 in germination percentages, the data was transformed by arc sine of the square root of the proportion to approximate the normal distribution and meet the statistical assumptions of normality and homogeneity.

RESULTS AND DISCUSSION

Number of seeds and fruit size

Of the six ripe fruits collected, 1275 black seeds were obtained at the physiological maturity stage. Seeds were found aggregated in clusters in the broadest part of the fruit (Figure 1A). The number of seeds per fruit was 334 as maximum and 97 as minimum with an average of 212 ± 34.2. Unripe seeds were yellow (Figure 1B). The wide difference between the maximum and minimum number of seeds per fruit has also been reported in other studies such as in *Echinocactus platyacanthus* where the number of seeds ranged from 100 to 620 with an average of 522 seeds per fruit (Ruíz-Pérez *et al*., 2021). In another Cactaceae, *Austrochthamalia teyucuarensis*, the number of seeds ranged from 13 to 99 (Duarte *et al*., 2019).

The averages were fruit length, 5.1 ± 0.37 cm and width, 1.55 ± 0.06 cm (Figure 1C). The number of seeds and the size of the fruit vary widely according to the species and the environmental conditions in which the plants develop, as well as the expression of various genes (Li *et al*., 2021).

Influence of scarification treatment and seed weight on germination.

The mechanical scarification treatment showed the best results, promoting the highest germination percentages in both seed groups. For group 1 seeds, germination was

93.2±3.6 % and for group 2 seeds 96±3.3 % (Table 1). It is important to emphasize that although the seeds were collected at physiological maturity and stored for one year at room temperature, they did not lose their viability. Garcia-Gonzalez *et al*. (2020) reported a lower germination percentage with 65 % in 1200 ripe seeds with mechanical scarification of *Echinocactus parryi.* Differences in germination percentages are probably due to collection time, storage time or seed weight. The mechanical scarification method is successfully used in the germination of other cactaceae. For example, in the species *Opuntia megacantha*, 100 % germination was obtained in shorter times than the control without scarification (González-Cortés *et al*., 2018). The mechanical scarification process is slow and laborious and must be done individually with each seed. However, following this process avoids damaging the embryo and allows the entry of water and oxygen to favour germination (Robles-Martínez *et al.*, 2016).

The 2 mg L^{-1} AG_3 treatment promoted 9.4 % germination in group 1 seeds and 6.8 % germination in group 2 seeds (Table 1). Results were not significant despite AG_3 is a hormone, widely used to promote germination, which allows activation of several genes involved in promoting embryo growth and development (Savaedi *et al*., 2019). In the cactaceae *Ferocactus histrix* and *Ferocactus latispinus*, in treatments with AG_3, germination percentages ranging between 35 and 37 % were obtained (Amador-Alferez *et al*., 2013).

In the case of *E. parryi* seeds treated with sulfuric acid, a germination percentage of 12.8±2.1 % was obtained for group 1 seeds. Whereas group 2 seeds showed 39.4±5.3 % germination and were statistically superior to the control treatment (Table 1). In other cactaceae species, the application of sulfuric acid presents significant effects by promoting 82 % germination in seeds of *Ferocactus pilosus* (Rodríguez-Ruiz *et al*., 2018). The difference in response could be related to the hardness of the seed coat; some seeds requiring passage through the digestive tract of an herbivore to dissolve or soften the seed coat (Koi *et al*., 2016). The seed mass in seed weight is very important

Table 1. Influence of the interaction between different scarification treatments and seed weight on the *in vitro* germination percentage of *Echinocactus parryi* 30 days after sowing.

Treatment	Seeds (Group)	Number	Germination percentage
Control	1	50	20.3 ± 3.4 bc
Control	2	50	25.6 ± 4.5 bc
Mechanic scarification	1	50	93.2 ± 3.6 a
Mechanic scarification	2	50	96.0 ± 3.3 a
AG_3 (2 mg L^{-1})	1	50	9.4 ± 1.9 c
AG_3 (2 mg L^{-1})	2	50	6.8 ± 1.64 c
H_2SO_4 (25 %)	1	50	12.8 ± 2.1 c
H_2SO_4 (25 %)	2	50	39.4 ± 5.3 b

Group 1: seeds weighing 35 to 45 mg; Group 2: seeds weighing 46 to 55 mg. Means ± standard error with different letters are statistically different according to Tukey's test ($p \leq 0.05$).

at the time of germination and seedling development. If different seed treatments are applied, germination can be more effective (Rodriguez *et al*., 2019). In general, mechanical scarification was the treatment that most influenced germination. It was possible to accelerate germination time by up to four times compared to the control group; thus, this is the best option to obtain *E. parryi* seedlings.

Morphology of *in vitro* germinated seedlings

Mechanically scarified seeds of *E. parryi* were used to analyse macroscopic changes. Figure 2A shows the appearance of the seed in which 50 % of the testa was removed, thus providing a greater entry of moisture into the embryo, which facilitates germination. From day 2, the emergence of green, succulent cotyledons, together at the base and separated at the end and triangular in shape, was observed (Figure 2B). By day 15, the seedling showed an intense green colour with white dots all over the epidermis and the root began to thicken (Figure 2E). From day 20 on, between the cotyledons, the presence of the epicotyl was evident with the emergence of trichomes. The intersection of the hypocotyl-root showed a brown to reddish coloration (Figure 2F), the same characteristic reported in two-week-old seedlings of *Neobuxbaumia mezcalaensis* and *S. queratoensis* species (Loza-Cornejo and Terrazas, 2011).

At 35 days, the cotyledons are open to the sides and in the apical part there are four tubercles, each with an areole, elongated spines and trichomes. At this stage the root has not yet branched; the seedling reached a height of 9 mm and a diameter of 4 mm (Figure 2G). After six months of *in vitro* culture, the epicotyl began to take on a rounded, succulent shape with scattered areoles and cotyledons were observed to be wilted (arrow). The stem had a height of 12.2 mm and diameter of 5.2 mm, the taproot was thickened and had a length of approximately 24 mm without branching (Figure 2H). At 12 months the stem was 19.1 mm high and 6.7 mm in diameter, had 4 rows of tubercles with 5 areoles in each one with 3 to 4 white thorns, which were vertically arranged in ribs. The root reached a length of 30 mm and is branched at the end (Figure 2I).

The morphological characteristics are adaptations to arid environments (Loza-Cornejo *et al.*, 2003), which can be appreciated from the juvenile stages, with the beginning of the development of thorns. Adult plants are globose, approximately 40 cm tall and have 13 spiral ribs with abundant thorns (Figure 3A); the flowers are yellow, appeared in summer and the diameter was about 7 cm (Figure 3B). The morphological description of *E. parry* seedlings is of great importance, because it has different characteristics from adult plants, which should be considered for taxonomic studies. Furthermore, it is necessary to know how the seedlings develop *in vitro* in order to extrapolate this information and hypothesize how the establishment would be in their natural ecosystem or at greenhouse propagation. In this way, their age in the juvenile stages would be approximated for the conservation of the species.

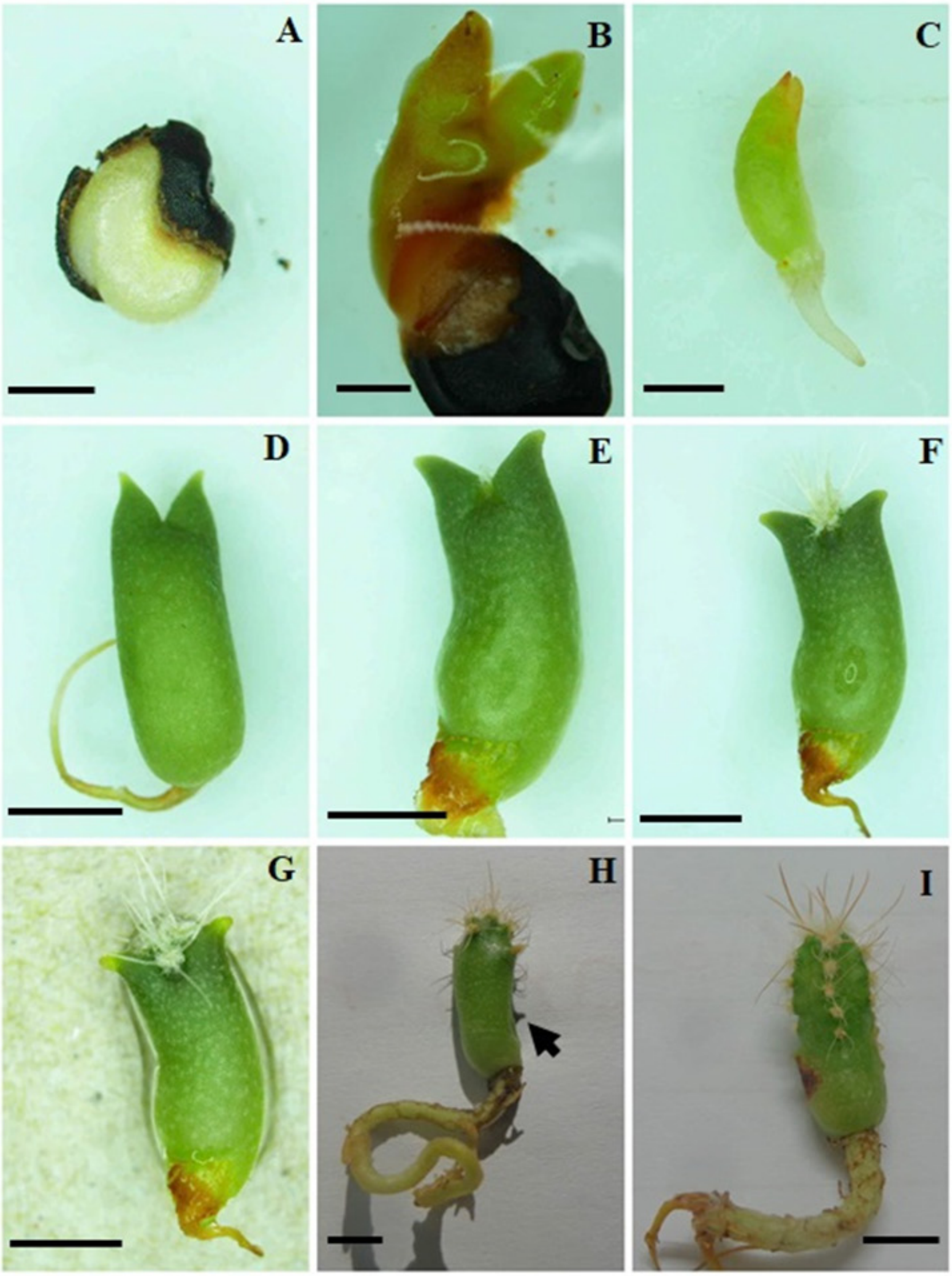

Figure 2. Morphology of *Echinocactus parryi* seedlings at different ages. A: scarified seed at day 1 (3X), bar represents 500 μm; B: appearance of cotyledons at day 2 (2X), bar represents 1000 μm; C: radicle emergence day 8 (1X), bar represents 5 mm; D: defined root and epicotyl formation at day 10 (1X), bar represents 5 mm; E: epicotyl thickening at day 15 (1X), bar represents 5 mm; F: emergence of trichomes at day 20 (1X), bar represents 5 mm; G: thorn formation at day 35 (1X), bar represents 5 mm; H: appearance of seedling at 6 months, bar represents 5 mm; I: appearance of plant at 12 months, bar represents 1 cm.

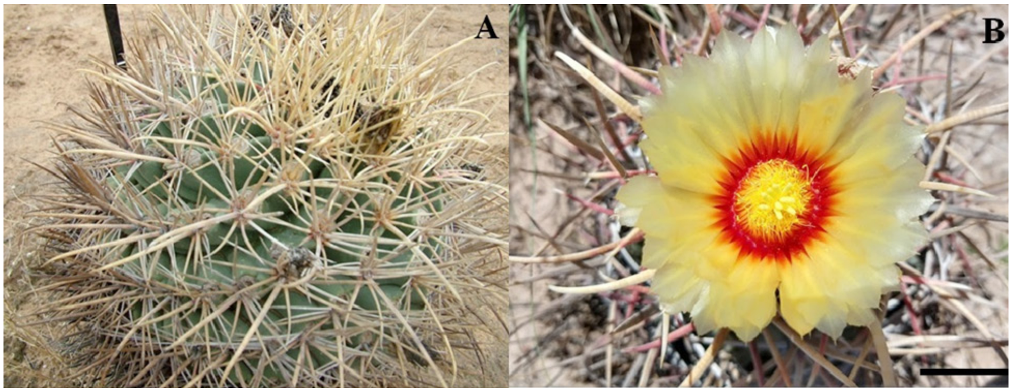

Figure 3. Adult specimen of *Echinocactus parryi* growing in its natural ecosystem. A: mature and seed-producing plant (photo: Pedro Osuna Ávila); B: flower of *E. parryi*, the bar represents 2 cm.

CONCLUSIONS

Scarification treatments applied to *Echinocactus parryi* seeds in this study indicated that mechanical scarification increases the germination potential of this species. Mechanical scarification was able to increase the germination percentage up to four times compared to the control (intact seeds). The description of the morphological characteristics in juvenile stages of *E. parryi* is an important contribution that can support taxonomic studies and the conservation of this species in juvenile stages. The information obtained can be extrapolated to know how plants become established in their natural ecosystem, from the beginning of germination, which is the most critical stage for the survival and development of plants up to a juvenile stage.

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