In vitro regeneration of the endangered cactus *Turbinicarpus mombergeri* (Riha), a hybrid of *T. laui* × *T. pseudopectinatus*

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**Abstract**

*Turbinicarpus mombergeri* is a cacti species formed by a hybridization process between *Turbinicarpus laui* and *Turbinicarpus pseudopectinatus*. Under natural conditions, it is very difficult for two species be genetically compatible for hybridization, and to produce flowers at the same time. Thus, *T. mombergeri* is a very interesting and a rare species. Unfortunately, the current populations are decreasing and now it is considered critically endangered. The aim of this research was to develop a successful protocol for propagating *T. mombergeri* using the in vitro culture techniques. Seed disinfection was performed with Plant Preservative Mixture, and 80% of germination occurred at day 45 in Murashige-Skoog medium. The shoots were cut longitudinally, and the segments were transferred to media containing 2.22 or 4.44 µM benzyladenine to induce shooting. The generated shoots were highly hydrated, and presented abundant callus. The hyperhydricity was controlled by reducing salt medium concentration, by increasing calcium levels and by using polyethylenglycol. The reduction of callus was attained by adding tri-iodo benzoic acid. Vigorous and thick shoots were generated in medium containing urea, and rooting improved in the presence of 0.5 µM indoleacetic acid. Plantlets with normal morphology were obtained, and the survival rate of the plants in soil was 80%. The methodology developed represents an alternative for propagation of *T. mombergeri* under controlled conditions for commercial or conservation purposes.

**Key message**

The paper describes several approaches to avoid the hyperhydricity and callus formation, to improve quality of shoots and to increase the development of roots during the micropropagation process of the endangered cacti *T. mombergeri*.

**Keywords** *Turbinicarpus mombergeri* · Micropropagation · Endangered cacti · Hybrid

**Introduction**

The Cactaceae family is native to the American continent and comprises about 2000 species. The major diversity of cacti, however, is located in Mexico, with more than 600 species, of which 80% are endemic (Ortega-Baes et al. 2010).

Cacti are succulent plants well adapted to dry and desert-like conditions. Many of them possess globe-shaped stems, combining the highest possible volume for water storage with the lowest area for water loss by transpiration (Gibson and Nobel 1986). The cacti species are valued in the international market because of the beauty of their flowers and the characteristic morphology of the stems.

Unfortunately, the natural populations of cacti are decreasing because the devastation of their natural habitat and over-collection (Goettsch et al. 2015). According to the Convention on International Trade of Endangered Species (CITES 2015), 35 cacti species are included in Appendix I, among them, several species of the genus *Turbinicarpus*. Particularly, the native populations of *Turbinicarpus mombergeri* have suffered the negative effects of plant looting because they are unique and very uncommon in the wild (Sotomayor-Martín del Campo et al. 2004). This species is generated by the hybridization of *Turbinicarpus laui* (Fig. 1a) and *Turbinicarpus pseudopectinatus* (Fig. 1b).
Recently, Khan et al. (2020) investigated the genetic architecture of hybridization in four areas of eastern Brazil that contain Melocactus concinnus, M. ernestii, M. glaucescens, M. paucispinus, and M. zehntneri. They observed that the genomic introgression among these species is very low, which confirms that Melocactus maintain their genetic integrity with selection favoring parental genotypes. Thus, the case of T. mombergeri generation is rare considering that is difficult that two species be genetically compatible to hybridize and to produce flowers at the same time.

T. mombergeri is a semi-globose cactus and possesses elliptical areoles with most of the spines in the lateral position. Two variants in the spination pattern has been described, in which T. laui (Fig. 1c) or T. pseudopectinatus (Fig. 1d) characteristics predominate. This species grows in calcareous gypsum rocky soil surrounded by thick xerophilous scrub. A single locality with three areas of occupancy of approximately 10,000 m² is known. The natural population of T. mombergeri is estimated fewer than 250 adult individuals, therefore, it is considered critically endangered (CITES 2015). In addition, the T. mombergeri plants are usually taken from the natural habitat, reaching high prices on the international market (Sotomayor-Martín del Campo et al. 2004).

An alternative for propagating and preserving rare and threatened cacti are the in vitro culture techniques. By using this methodology, more than one hundred species have propagated, either by organogenesis (Lema-Rumińska and Kulus...
The aim of this research was to develop an efficient protocol for regenerating *T. mombergeri* in vitro and for contributing to its conservation.

**Materials and methods**

**Disinfection and germination of seeds**

The seeds of *T. mombergeri* were donated by the Instituto Nacional de Investigaciones Agrícolas y Pecuarias, in San Luis Potosí. The seeds (n = 20) were rinsed with water and commercial soap, maintained in tap water for 1 h and soaked 72 h in deionized water. They were immersed in 20 ml L⁻¹ Plant Preservative Mixture™ (Plant Cell Technology, Washington, DC, USA) for 24 h with constant agitation at 125 rpm. The seeds were germinated in MS medium (Murashige and Skoog 1962) supplemented with 116 μM Myo-inositol, 1.2 μM thiamine-HCl, 30 g L⁻¹ sucrose and 4 g L⁻¹ Phytagar (Sigma-Aldrich, St. Louis, MO, USA). The pH of the medium was adjusted to 5.7. The subcultures were performed every 30 days for 90 days. The pH medium was adjusted to pH 6.7 to obtain a final of pH 5.7 after sterilization. The percentage of germination was determined at 7, 14, 30, 45, 60 and 90 days, considering the emergence of radicle as positive germination.

**Induction of *T. mombergeri* shoots**

The seedlings germinated from seeds segmented longitudinally, and the apical tip was removed. The segments were cultivated in Murashige Skoog medium (MS) containing 2.22 μM (B2) or 4.44 μM (B4) benzyladenine (BA) and 1% activated charcoal (AC), and solidified with 8 g L⁻¹ Phytagar (Phyto Technology, KS, USA). The pH medium was adjusted to pH 6.7 to obtain a final of pH 5.7 after sterilization. The percentage of new shoots was evaluated at 30, 60 and 90 days. The presence of callus and hyperhydricity was recorded as: low (less than 10% of callus or hyperhydricity on the tissue surface), medium (between 10 and 30% of callus or hyperhydricity on the tissue surface) or high (more than 50% of callus or hyperhydricity on the tissue surface).

**Statistical analysis**

A completely randomized design was selected and significant differences between mean values were evaluated by ANOVA using the Tukey test with a 95% of significance level with the GraphPad Instat 3 program (GraphPad Software Inc., Version 3.10).

**Results and discussion**

**Shoot induction**

To initiate the in vitro culture of endangered species, the use of seeds is the preferred method because it avoids destroying the mother plants and preserves genetic diversity. In this work, seed disinfection with PPM proved to be efficient, and no contamination was observed (data not shown). This
compound is a broad-spectrum biocide with no adverse effects on in vitro seed germination, callus proliferation or callus regeneration.

Although the germination is usually low in seeds with a hard coat (Rojas-Arèchiga and Vázquez-Yanes, 2000), as with T. mombergi, we obtained 80% of the germination at 45 days (Fig. 2a). Longer periods did not improve the response. The percentage of germination obtained in this study was higher than the data reported for T. laui with in vitro conditions (29%) or under ex vitro conditions for T. pseudopestinatus (48%) (Santos-Díaz et al. 2003; Flores et al. 2008). In addition, the germination rate was higher than that reported for Turbinicarpus valdezianus and T. subterraneus, which presented 46% and 90% of germination at double time (Dávila-Figueroa et al. 2005). The best response obtained in this work could be associated with the disinfection protocol. The asepsis of T. laui, T. valdezianus and T. subterraneus seeds was performed with sodium hypochlorite and ethanol, which are more aggressive agents than PPM, and therefore could damage the embryo structure. Other factors that affect germination in cacti are age, size, dormancy and origin of seeds (Rojas-Arèchiga and Vázquez-Yanes 2000; Gurvich et al. 2017).

After seed germination, well-defined epicotyls and roots were observed at 30 days, reaching 4 mm and 5.8 mm, respectively, at 90 days. The presence of spines was evident after 14 days and increased proportionally to time culture (Fig. 2b). Since T. mombergeri is a little known species, there is no information about the germination rate or growth parameters in the wild for comparison.

The epicotyls of T. mombergeri were cut longitudinally, and the segments were transferred to a medium with BA. Because the scarcity of material (14 germinated seeds) only B2 and B4 media containing AC were tested. These BA concentrations were selected because, in previous studies, they successfully induced the shoot formation in T. laui (Santos-Díaz et al. 2003); it also has been reported that BA was efficient in propagating other Turbinicarpus species (Pérez-Molphe-Balch et al. 2015). The AC was included on media because is well known that this compound decreases toxic metabolites present in the medium, and adsorbs phenolic compounds related to the explant browning (Thomas 2008). Data showed that 50% and 7% of the explants cultivated on B2 and B4 media, respectively, regenerated one shoot at 90 days, which were highly hydrated, and presented abundant callus formation (Table 1, Fig. 3a). This response was lower than that reported by Dávila-Figueroa et al. (2005), who obtained between 7.8 and 19.7 shoots per explant during the propagation of several Turbinicarpus species. It

![Fig. 2 Germination and development of seedlings of Turbinicarpus mombergeri.](image)

**Table 1** Induction of Turbinicarpus mombergeri shoots in medium with benzyladenine at 90 days

| Medium | Shooting<sup>a</sup> (%) | Hyperhydricity<sup>b</sup> (%) | Callus<sup>b</sup> (%) |
|--------|--------------------------|-------------------------------|-----------------------|
|        | 0 | Low | Medium | High | 0 | Low | Medium | High | 0 | Low | Medium | High |
| MS-B2  | 50 a | 0 | 28 | 28 | 44 | 14 | 28 | 44 | 14 |
| MS-B4  | 7 b | 0 | 0 | 0 | 100 | 0 | 100 | 0 | 0 |

<sup>a</sup>Means with different letter differed significantly (Tukey test, p ≤ 0.05)

<sup>b</sup>Low: less than 10% of callus or hyperhydricity on the tissue surface; medium (10–30%) of callus or hyperhydricity on the tissue surface; high > 50% of callus or hyperhydricity on the tissue surface

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has described that the heterosis in hybrids can affects the regenerative capacity. For example, the ability for generating in vitro shoots was higher in a tomato parental line than in their hybrids, and this difference was attributed to the heterosis and maternal effects (Ohki et al. 1978). Additional genetic studies must be done to determine if this phenomena is also present in the hybrid *T. mombergeri*.

The shoots were transferred to B2 to increase shoot number, and after a second subculture an average of 2.8 shoots per explant were obtained, still hydrated and with abundant callus. Hyperhydricity have been described during micropropagation of many cacti species, such as, *Mammillaria gracillis*, *M. pectinifera*, *Escobaria minima* and *Pelecyphora aselliformis*, among others (Giusti et al. 2002; Poljuha et al. 2003). This effect has often been considered a physiological response to simultaneous stress factors of the in vitro culture, which negatively impacts the micropropagation efficiency and survival of plants in ex vitro conditions (Bayraktar et al. 2020). Some biochemical characteristics present in hyperhydric tissues are reduced dry weight, and less lignin, cellulose and calcium content, as well as a low calcium/uronic acid ratio (Kevers et al. 2004).

Hyperhydricity can be reduced by improving ventilation and to decrease ethylene accumulation in vessels; by adding osmotic agents (mannotol, polyethylene glycol), to diminish the water potential of media and to low the water content in tissues; by decreasing the concentration of nutrients in the medium; or by increasing the calcium concentration (Thomas et al. 2000; Snyman et al. 2011; Nikam et al. 2019).

Therefore, to reduce the hyperhydricity in *T. mombergeri* shoots, the effect of culture media (MS, 1/2 MS, 1/4 MS, 1/2 WPM media), osmotic agents (1% PEG) and double calcium concentration (2Ca) were tested. The reduction in salts concentration in 1/2 MS medium generated 21% of compact shoots at 90 days. This percentage improved in 1/4 MS medium or 1/2 WPM medium containing 2Ca concentration and PEG, generating between 80 and 90% of compact shoots at 90 days. A reduction on compact shoots, however, was lightly increased and longer roots (4.6 mm) were generated at 90 days. A reduction on compact shoots, however, was lightly increased and longer roots (4.6 mm) were generated at 90 days.

On the other hand, 100% of shooting was observed in 1/2 WPM-2Ca-P medium (Table 2) generating two shoots per explant. Although the formation of compact shoots was attained, the presence of callus was still very high as shown in Fig. 3b.

Several approaches have been used to reduce callus formation, including cytokinin elimination or the employment of auxin transport inhibitors, such as TIBA. This compound enhanced somatic embryogenesis in groundnut and shoot formation in *Morus alba* (Bhua and Wakhlu 2001) and enhanced root formation in *Echinocactus parry* (García-González et al. 2020). Thus, we cultivated the *T. mombergeri* shoots in 1/2 WPM-2Ca-P added with 0.5, 1 and 2 mg L⁻¹ TIBA. The callogenesis was reduced in the presence of TIBA proportionally to the concentration (Table 3). This result suggests that the *T. mombergi* shoots must synthesize high levels of endogenous auxins that are responsible for callus generation. Figure 3c shows the aspect of *T. mombergeri* shoots without callus at 90 days of culture.

### Root formation and transfer to soil

The compact shoots (2 to 3 cm high) were transferred to media 1/2 WPM-2Ca-P medium with 1 mg L⁻¹ TIBA (named WCPT) alone or in combination with 5.7 µM IAA (WCPT-1) or 0.5 mg L⁻¹ urea (WCPT-2) to induce the rooting of shoots (Table 4). After 90 days in the WCPT medium, 21.7% of the explants developed roots of approximately 3.8 mm long. In WCPT-1 medium, the percentage of rooting lightly increased and longer roots (4.6 mm) were generated at 90 days. A reduction on compact shoots, however, was observed according to time, probably because of the presence of the auxin in the medium, which induced an incipient callus formation.

The shoots cultivated in media WCPT-2 generated the lowest percentage of rooting, the root length was similar to that obtained in the WCPT-1 medium, but the shoots duplicated their diameter at 90 days (Fig. 3d).
Taking into account these results, an additional experiment was performed (WCP-3). The shoots were maintained in the medium WCPT-2 for 90 days to generate wide and thick shoots. The plant material was then transferred to medium WCPT-1 for 60 days, to induce a vigorous radical system, and was finally maintained in the WCP medium for 120 days (Table 4). Using this strategy, the callus formation was avoided completely, and at the end of experiment 96% of rooted shoots were generated with well-defined roots from an average length of 13 mm. Figure 3e shows the aspect of rooted shoots after 1 year in culture. These results show that *T. mombergeri* requires a long period to develop strong roots. In wild conditions, most *Turbinicarpus* species exhibit a very thick primary root, which represents 80% of the plant body, and acts like an anchor, and more importantly, as water storage for dry periods. The root growth is therefore a time-consuming event.

The beneficial effect of urea in growth and rooting of *T. mombergeri* shoots is attributed to a higher availability and better absorption of organic nitrogen. It is well known that nitrogen is required for the synthesis of chlorophyll and for amino acid metabolism, which are essentials for plant growth and development. Several urea transporters have been identified across different cellular membranes. For example, in *Arabidopsis*, a symporter, that cotransports urea with protons at high affinity, has been described. In the tonoplast, various tonoplast intrinsic proteins (TIPs), a subfamily of aquaporins, transport urea in a channel-like manner. These transporters seem to optimize the nitrogen intake and compartmentation in dependence of the nitrogen forms being available in the medium (Kojima et al. 2006). Further studies must be done to identify the putative urea transporters in *Turbinicarpus* species.

At the end of this research, about 650 shoots were obtained from 14 germinated seeds, representing a 46-fold increase in relation to seed propagation, indicating a good efficiency of the micropropagation process.

The *T. mombergeri* plants were transferred to soil, and 85% survived after 1 year. At this period, the plants showed the characteristic spines pattern observed in mature plants (Fig. 3f).

In summary, this work shows that reduction of salt medium concentration, high level of calcium concentration and presence of PEG reduced the shoots hyperhydricity. The addition of TIBA decreased caulogenesis and the presence of urea promoted the development of thick shoots. The protocol developed allowed the successful micropropagation of

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**Table 2** Effect of medium components in hyperhydricity of *Turbinicarpus mombergeri* shoots at 90 days

| Medium*                  | n  | Shoots per explant | Shooting (%) | Hyperhydricity (%) |
|--------------------------|----|--------------------|--------------|--------------------|
| MS                       | 28 | 0.25 d             | 25           | 0                  |
| ½ MS                     | 28 | 1.39 b             | 86           | 14                 |
| ¼ MS-2Ca-P               | 41 | 0.39 d             | 39           | 21                 |
| ½ WPM-2Ca                | 24 | 0.79 c             | 79           | 34                 |
| ½ WPM-2Ca-P              | 33 | 2.12 a             | 100          | 41                 |

1/2 MS MS medium at 50% salt concentration, 1/4 MS MS medium at 25% salt concentration; 1/4 MS-2Ca-P 1/4 MS medium with double calcium concentration and 1% PEG; 1/2 WPM-2Ca WPM medium at 50% salt concentration with double calcium; 1/2 WPM-2Ca-P 1/2 WPM medium with double calcium and 1% PEG

All media contained 1% activated charcoal. Values with different letter are statistically different (Tukey test, p < 0.05)

**Table 3** Effect of TIBA on callus formation of *Turbinicarpus mombergeri* shoots at 90 days

| TIBA concentration (mg L⁻¹) | n  | Shoots per explant | Callus formation (%) |
|-----------------------------|----|--------------------|----------------------|
| 0.5                         | 36 | 1.36 b             | 38.8                 |
| 1                           | 46 | 1.65 b             | 41.3                 |
| 2                           | 50 | 1.88 b             | 82                   |

The medium used was 1/2 WPM with double calcium concentration and 1% PEG, pH 5.7

Values with different letter are statistically different (Tukey test, p < 0.05)
the critically endangered cacti *T. mombergeri*, contributing to its conservation.

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**Author contributions** MLSD realized the propagation of shoots, the experiments focused on reduction of hyperhydricity and callus formation, and the rooting of shoots. JAR participated in the germination of seeds and induction of *T. mombergeri* shoots. MSSD is the leader of the group, designed the project and experimental work, participated in revision, discussion of results, wrote the paper and elaborated tables and figures.

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**Data availability** Data and material are available at the Faculty of Chemistry, UASLP.

**Code availability** The software used was Microsoft Word.

**Declarations**

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethical approval** No animals or persons were used in this work.

**Consent to participate** MLSD, JRA and MSSD give their consent to participate in this paper.

**Consent for publication** MLSD, JRA and MSSD give their consent for the publication of this paper.

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### Table 4 Rooting of *Turbinicarpus mombergeri* shoots

| Treatment        | Time (days) | Compact shoots (%) | Rooting (%) | Root length (mm) |
|------------------|-------------|--------------------|-------------|------------------|
| WCPT (Control)   | 30          | 75                 | 0           | –                |
|                  | 60          | 91                 | 0           | –                |
|                  | 90          | 100                | 21.7        | 3.8 ± 0.08       |
| WCPT-1           | 30          | 82                 | 0           | –                |
|                  | 60          | 73                 | 26          | 3.8 ± 0.07       |
|                  | 90          | 69                 | 26          | 4.6 ± 0.06       |
| WCPT-2           | 30          | 52                 | 0           | –                |
|                  | 60          | 73                 | 17          | 3.5 ± 0.05       |
|                  | 90          | 100                | 17          | 4.2 ± 0.05       |
| WCPT-3           | 90 (WCPT-1) | 92                 | 60          | 8.75 ± 0.3       |
|                  | 60 (WCPT-2) | 96                 | 70          | 10 ± 0.4         |
|                  | 120 (Control)| 96                | 96          | 13 ± 0.7         |

WCPT ½ WPM medium with double calcium concentration, 1% PEG, 1 mg L⁻¹ TIBA, WCPT1 WCPT medium with 5.71 µM IAA, WCPT-2 WCP medium with 0.5 mg L⁻¹ urea, WCPT-3 shoots were maintained in WCPT-2 medium for 90 days, transferred to WCPT-1 for 60 days and to WCPT medium for 120 days.

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