Article

Stability of the explant in the *in vitro* proliferation of axillary shoots of the biznaga

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Abstract

To conserve *ex situ*, promote the use and enhancement of native germplasm of ornamental interest such as *T. viereckii* subsp. major, a species at risk Pr, the present research was proposed with the aim of regenerating this species in the Plant Tissue Culture Laboratory (LCTV) of the Saltillo CIRNE-INIFAP Experimental Field considering the stability of the explant during subsequent subcultures, determine the type and concentration of phytohormones and the best cytokinin-auxin ratio that most influence the *in vitro* multiplication stage. The intermediate segments of the stem (SIT) obtained from vitroplants was used as an explant. From 2017 to 2019, the multiplication stage was evaluated using the Murashige-Skoog medium (MS; Murashige and Skoog, 1962) at 50% of its macrosalts. A completely randomized experimental design with factorial arrangement was used to evaluate two cytokinins: 6-benzyl aminopurine (BAP) and 6-furfuryl aminopurine (KIN) at four concentrations 2.5, 5, 7.5 and 10 mg L^{-1} in interaction with two auxins: naphthaleneacetic acid (NAA) and indole butyric acid (IBA) in a cytokinin-auxin ratio of 10:1. In total, 16 treatments were evaluated, establishing five SIT/bottle with 10 repetitions/treatment. This evaluation was repeated five times by means of subcultures, evaluating the number of shoots/explant (*Nb*) and the shoot height (*Ab*, mm) every ten weeks. The analysis of variance (Anova) showed that there was stability of the explant during the subcultures, maintaining a similar multiplication rate for more than three years, an important effect for intensive multiplication. When analyzing the treatments as independent effects in the Tukey mean test ($p \leq 0.05$), the interactions BAP: IBA and KIN: IBA with concentrations of 5 mg L^{-1} BAP + 0.50 mg L^{-1} IBA and 2.5 mg L^{-1} KIN + 0.25 mg L^{-1} IBA were selected as the treatments that induce shoots with a multiplication rate of 9.25 shoots/explant with an *Ab* of 6.73 mm.

Keywords: *in vitro* culture, growth regulators, ornamental cacti.

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Introduction

The advance in the knowledge of *in vitro* hormonal regulation has allowed the development of micropropagation protocols in species and cultivars of economic importance, such as in hybrid rose *Mimosa pudica* L. (Bianchetti *et al*., 2017); (Aggarwal *et al*., 2018), even in red algae (Villanueva *et al*., 2013). The effect of different concentrations of phytohormones on the micropropagation of species of the family Cactaceae has been studied in germplasm of interest for ornamental horticulture at risk status, such as: in several species of the genus *Coryphanta* spp. (Pérez-Molphe-Bach *et al*., 1998), the old man cactus *Cephalocereus senilis* (Haw.) Pfeiff. (Choreño *et al*., 2002), swallow nest biznaga *Epithelantha micromeris* (Engelm.) F. A. C. Weber ex Britt. & Rose (Villavicencio *et al*., 2012), mammillaria *Mammillaria voburnensis* Scheer (Ordoñez, 2003), artichoke cactus *Obregonia denegrii* Frič & A. Berger (Cardarelli *et al*., 2010), various mini cactus of the genus *Turbinicarpus* spp. (Mata-Rosas *et al*., 2001; Dávila *et al*., 2005; Villavicencio *et al*., 2011) and pitaya *Stenocereus stellatus* (Pfeiff.) Riccob. (Martínez-Villegas *et al*., 2011), where *in vitro* shoots have been obtained using cytokinins alone or combined with auxins.

There are results with the interaction of two cytokinins 6-benzyl aminopurine (BAP) and 2-isopentenyl-adenine (2-iP), in combination with low levels of auxins; in other cases, with the interaction of cytokinin-auxin such as: BAP-α-naphthaleneacetic acid (NAA), BAP-indole-3-butyric acid (IBA), 6-furfuryl aminopurine (KIN)-NAA or indole-3-butyric acid (IBA). This variety of responses is a function of the genotype and culture medium used. Gonçalves *et al*. (2016) mention that cytokinins (CK) and auxins (AUX) have been used to regulate the growth and development of plant tissues, but it is not yet clear how these compounds influence the metabolic activity of species *in vitro*, where CK concentrations from 0 to 20 μM can influence the functionality of the photosystem (PS) II, leaf anatomy, stomatal density, production of shoots from meristems or axillary buds.

Considering that the *in vitro* culture of plant tissues has become a very useful tool to accelerate plant production processes, its feasibility will depend on the management of the factors that favor this process, such as the culture medium used, where the concentration of nutrients, hormones, hours of light, temperature, pH, relative humidity, oxygen supply, among others, influence the development of plants; so the morphogenetic response of the explant will depend on the standardization of the micropropagation protocol.

With the purpose of carrying out *ex situ* conservation actions, promote the use and enhancement of native material of ornamental interest (Espinoza-Flores *et al*., 2003; Granada, 2014; Gámez *et al*., 2016), such as the ornamental biznaga 'inverted cone of Viereck' *Turbinicarpus viereckii* subsp. major (Glass & R.A. Foster) Glass, native to northern Mexico, belonging to the subfamily Cactoideae and Tribe Cacteae (Guzmán *et al*., 2003; Hunt, 2006; Tropicos, 2019) and that it is in special protection risk status (Pr) according to NOM-059-ECOL-2010 (SEMARNAT, 2010), it became necessary to evaluate other unconventional propagation schemes to regenerate this species through *in vitro* culture, proposing as an objective of this research to define the type and dose of cytokinins, as well as the cytokinin-auxin ratio that influence the multiplication stage, to select the ratio of phytohormones that promotes the greatest number of shoots *in vitro* without losing the stability of the explant and standardize the micropropagation protocol to regenerate this species and produce plants in sufficient quantities.

Materials and methods

The research was carried out in the facilities of the Plant Tissue Culture Laboratory (LCTV) of the Saltillo CIRNE-INIFAP Experimental Field, located in the municipality of Saltillo, Coahuila, Mexico.

Plant material. In the first stage, vitroplants germinated *in vitro* from seed collected in the summer of 2016 in the municipality of Guadalcázar in San Luis Potosí were used.

Explants. When the vitroplants reached a height of 10 mm ±1, two cuts were made to remove the distal and proximal area of the stem, using the intermediate segments of the stem (SIT) as explants. This type of explant was used in all subsequent subcultures (Figure 1).

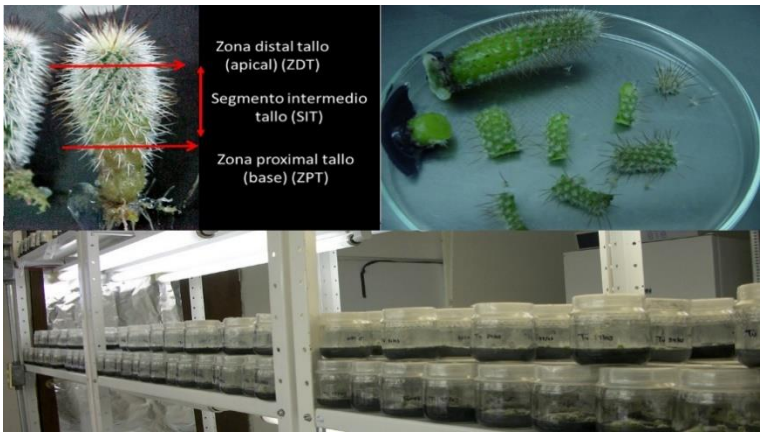

Figure 1. Obtaining explants and establishing treatments in the multiplication of the ornamental biznaga 'inverted cone of Viereck' (*Turbinicarpus viereckii* subsp. major (Glass & R. A. Foster) Glass).

From the second subculture, the SITs obtained from the distal area of the stem (ZDT) (apical) were used, which were subcultured and grown in the Murashige-Skoog medium (MS; Murashige and Skoog, 1962) as a base medium, halving (50%) its concentration of macrosalts and without growth regulators, to obtain again intermediate segments of the stem (SIT) and produce the required explants in the following subcultures and continue with multiplication.

Multiplication of shoots. The intermediate segments of the stem (SIT) used as explants were established in the Murashige-Skoog medium (MS; Murashige and Skoog, 1962) at 50% of its macrosalt concentration, supplemented with 100 mg L^{-1} of myo-inositol (SIGMA ®), 0.4 mg L^{-1} of thiamine-HCl (SIGMA®, T-3906), 1 mg L^{-1} of pyridoxine-HCL (SIGMA®, P-8666), 30 g L^{-1} of sucrose (SIGMA®, K-0753) and 7 g L^{-1} of agar (SIGMA®, A-1296), with 200 mg L^{-1} of activated charcoal. The pH of the medium was adjusted to 5.7 and previously sterilized in a Felisa brand autoclave at 1.2 kg cm^{-2} pressure at 121 °C for 15 min.

Using a completely randomized experimental design with factorial arrangement, two cytokinins were evaluated: BAP and KIN at four concentrations 2.5, 5, 7.5 and 10 mg L^{-1} in interaction with two auxins: NAA and IBA in a cytokinin-auxin ratio of 10:1. Five SITs per bottle were established,

with 10 repetitions per treatment and a total of 16 treatments were evaluated. This evaluation was repeated five times by successive subcultures from 2017 to 2019 in the LCTV, evaluating the number of shoots per explant (*Nb*) and the shoot height (*Ab*, mm) every ten weeks.

Statistical analysis. The evaluated variables were analyzed using the PROC GLM procedure of the statistical analysis system SAS, (Version 9.4) (SAS, 2019), performing an analysis of variance (Anova) to determine the effect of phytohormones (cytokinins and auxins), influence of concentration and a Tukey multiple range test ($p \leq 0.05$) considering the treatments as independent effects to select the best treatment.

Incubation conditions. The vegetative material was established in the incubation room of the LCTV considering a photoperiod of 14 h light by 10 of darkness, with a luminous intensity of 9-10 μmol sec^{-1} m^{-2} and a temperature of 25 ±2 °C.

Acclimatization and transfer to soil. The shoots obtained were individualized and subcultured in a MS medium at 50% of its components, adding 1 g L^{-1} of activated charcoal to induce rooting and then passed to their acclimatization. The rooted vitroplants were washed and treated with 1 g L^{-1} of commercial systemic fungicide with active ingredient N-(trichloromethylthio) cyclohex-4-ene-1,2-dicarboximide prior to transplantation in pots of 0.15 L capacity (6.5 x 5.6 x 5.6 cm). As a substrate, a mixture sterilized in an autoclave (Sumi Brand) at a temperature of 120 °C and 1.5 pounds of pressure, composed of sand + peat moss + agrolite in a 3:2:1 ratio, was used.

Results and discussion

In vitro culture stability. Although, there were significant differences (F= 60.31, p= 0.0001) between evaluations in the Anova, the statistical parameters corresponding to a high coefficient of determination (R^2) of 0.72, lower coefficient of variation (CV) of 26.4 and minimum significant difference (MSD) of 0.44 showed that the intermediate segments of the stem (SIT) used as an explant can express a morphogenetic response under the influence of the treatments.

Under *in vitro* conditions, 1 to 22 shoots per explant can be obtained, with subcultures III and IV where, on average, an *Nb* of 9.22 ±1 shoots per explant was obtained (Figure 2a). Although there were differences between the first and last of the five subcultures, the results were relevant for a mass production system, which can be maintained for more than three years and decrease its production from the fifth clonal multiplication, an issue that must be considered in the programming of production on a commercial scale.

In shoot height (*Ab*), significant differences between evaluations (F= 427.45, p= 0.0001) were also found, registering a coefficient of determination (R^2) of 0.50, a coefficient of variation (CV) of 32.65 and a minimum significant difference (MSD) of 0.25. Under *in vitro* conditions, the *Ab* ranges from 1 to 18 mm, with subcultures III and IV where, on average, an *Ab* of 7.56 ±1 mm was obtained; however, the size of the shoots obtained in all the evaluations carried out could be manipulated by the operator when performing the subculture (Figure 2a).

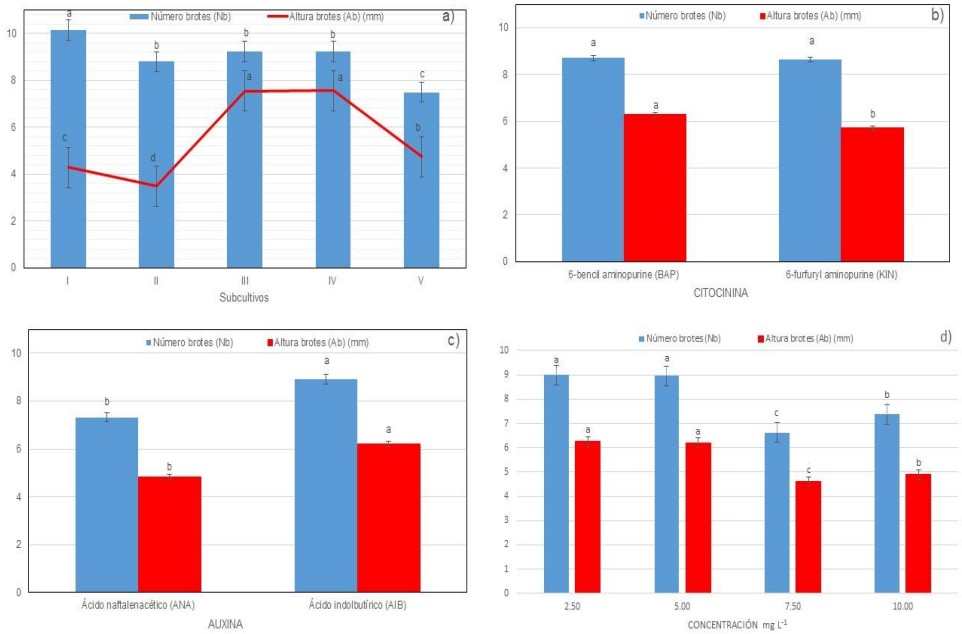

Figure 2. Number of shoots per explant1 (*Nb*) and shoot height (*Ab*) in the *in vitro* multiplication of the ornamental biznaga 'inverted cone of Viereck' (*Turbinicarpus viereckii* subsp. major (Glass & R. A. Foster) Glass). a) influence of the stability of the *in vitro* subculture; b) influence of the type of cytokinins; c) influence of the type of auxins; and d) influence of concentration.

The results recorded in each of the evaluations carried out showed that the SIT explants are stable in *in vitro* culture, being an organized plant tissue with axillary buds, from which the biznaga 'inverted cone of Viereck' *T. viereckii* subsp. major can be regenerated.

Influence of the type of cytokinins. When analyzing the total of the subcultures, Anova does not show significant differences ($p \leq 0.05$) between the cytokinins evaluated in *Nb*; however, with the culture medium MS at 50% with KIN, the greatest response was obtained, registering on average 8.69 shoots per explant in *Nb* (Figure 2b).

The multiplication rate for the biznaga *T. viereckii* subsp. major with both cytokinins exceeds that obtained in *M. craigii* (4.65), *M. formosa* (4.42), *M. obscura* (4.78) and *M. uncinata* (5.25) (Pérez-Molphe-Balch *et al*., 1998), *M. oteroi* (5.3) (Castro-Gallo *et al*., 2002) and is like that reported for biznaga 'inverted cone of Knuth' (*Turbinicarpus knuthianus* (Boed.) John and Říha) (Villavicencio *et al*., 2011). In *Ab*, there was a statistically significant difference ($p \leq 0.05$) between cytokinins, where BAP is the phytohormone that influences this variable with an *Ab* of 6.27 mm, exceeding in size the shoots obtained with KIN (Figure 2b).

In other species of cacti such as *Epithelantha micromeris* var. *micromeris*, *Strombocactus disciformis*, and *T. schmiedickeanus* var. *klinkerianus* (Soltero and Portillo, 2015) and *T. knuthianus* (Villavicencio *et al*., 2011), KIN has also been used for *in vitro culture*, as in other ornamentals, such as orchids *Laeliocattleya* (Orchidaceae) (Gonçalves *et al*., 2016) and coffee (*Coffe arabiga*) (Cantos-Cevallos *et al*., 2018), showing that the morphogenetic response depends

on the cytokinin used and the genotype. KIN is of natural origin, derived from purines or adenines, and can produce an effect on cell division and induce a morphogenic response in the explant, activating the axillary buds or areolar meristems that induce the formation of shoots of the biznaga *T. viereckii* subsp. major as has also occurred in *Mammillaria* schiedeana schiedeana (Soria-Campos *et al*., 2013).

Influence of the type of auxins. There were significant differences ($p \leq 0.05$) between the auxins evaluated, with indole butyric acid (IBA) being the one that influenced morphogenetic response in both *Nb* and *Ab.*

With this auxin, a greater response in *Nb* was obtained, registering on average 8.92 shoots per explant, with an *Ab* of 6.20 mm, exceeding the effect generated with naphthaleneacetic acid (NAA), where both variables registered a lower average, with an *Nb* of 7.3 shoots per explant and an *Ab* of 4.5 mm (Figure 2c).

IBA has also been used for the induction of shoots of *Epithelantha micromeris* var. *micromeris*, where up to 13 shoots per explant have been obtained (Villavicencio *et al*., 2012), while the use of NAA has had an effect on other cacti of the genus *Mammillaria* spp. (Pérez-Molphe-Balch *et al*., 1998; Castro-Gallo *et al*., 2002; Giusti *et al*., 2002; Ramírez-Malagón *et al*., 2007; Soria-Campos *et al*., 2013), registering a height similar to that reported in this paper.

The greater number of shoots with smaller size is an effect that frequently occurs in micropropagation, as reported by Mata-Rosas *et al*. (2001); Pérez-Molphe-Balch *et al.* (2015), so it is always appropriate to evaluate various cytokinin-auxin combinations to determine the balance between the response variables that are sought in the *in vitro* multiplication stage of the species of interest. This to ensure intensive propagation, give continuity to the multiplication stage and ensure survival during acclimatization.

Influence of concentration. Regardless of the type of cytokinin used, there were highly significant differences between concentrations ($p \leq 0.01$). The concentrations of 2.5 and 5 mg L^{-1} were statistically equal, surpassing the rest of the concentrations evaluated, with an *Nb* of 8.99 shoots/explant and an *Ab* of 6.24 mm (Figure 2d).

Influence of the type of concentration and cytokinin. The 50% MS base medium added with 2.5 mg L^{-1} of KIN and with 5 mg L^{-1} of BAP were statistically equal, surpassing the rest of the concentrations evaluated. Both cytokinins and concentrations promoted shoot induction with an *Nb* of 9.19 and 9.09 shoots per explant, respectively (Table 1).

The difference between both concentrations and type of cytokinins was in the height of shoots, where the greatest response was obtained with 5 mg L^{-1} of BAP, registering an *Ab* of 6.55 mm. These results showed that SITs were more compatible with KIN, a natural phytohormone, where a lower concentration in plant tissue generated a greater response in the induction of shoots, unlike SITs that were exposed with BAP, which is a synthetic cytokinin, where the greatest induction of shoots was obtained by doubling the concentration (Table 1).

Table 1. Type of cytokinin and concentration in *in vitro* multiplication of the ornamental biznaga 'inverted cone of Viereck' (*Turbinicarpus viereckii* subsp. major (Glass & R. A. Foster) Glass).

Cytokinin	(mg L^{-1})	Number of shoots (*Nb*)		Shoot heigh (*Ab*)	
		Mean	Standard deviation	Mean	Standard deviation
6-benzyl aminopurine (BAP)	2.5	8.78	±3.25	6.7	±3.02
	5	9.09	±4.21	6.55	±2.8
	7.5	7.24	±3.65	4.72	±1.09
	10	8.03	±4.46	4.86	±1.86
6-furfuryl aminopurine (KIN)	2.5	9.19	±3.58	5.85	±2.35
	5	8.83	±3.33	5.85	±2.68
	7.5	5.62	±2.34	4.44	±1.18
	10	6.45	±2.57	4.92	±1.29

The effect of the type of cytokinin and concentration on the number and height of shoots has also been reported in globose-type biznagas, where 3 to 10 mg L^{-1} of KIN (Soltero and Portillo, 2015) and from 0.5 to 2 mg L^{-1} have been applied when BAP has been used (Soria-Campos *et al.* 2013).

The results show that hormonal control influences the differentiation of the explant, as reported by Pérez-Molphe-Balch and Dávila-Figueroa (2002); Dávila *et al*. (2005); Ascough and Van Staden (2010); De la Rosa-Carrillo *et al*. (2012). This control had a positive effect on *in vitro* culture, making *ex situ* conservation feasible for both the cactus under study *T. viereckii* subsp. major, and for other species, such as the old man cactus *Cephalocereus senilis* (Haw.) Pfeiff. (Choreño *et al*., 2002), pitaya *Stenocereus stellatus* (Pfeiff.) Riccob. (Martínez-Villegas *et al*., 2011), mammillaria *Mammillaria voburnensis* Scheer (Ordoñez, 2003), artichoke cactus *Obregonia denegrii* Frič & A. Berger (Cardarelli *et al*., 2010) and mini cactus *Turbinicarpus* spp.

Influence of treatments. When performing the Tukey mean test ($p \leq 0.05$) to the treatments as independent effects, considering the interaction between the type of cytokinin and auxin with their different concentrations evaluated, it was determined that there are two treatments that can be used in the multiplication stage. The 50% MS medium added with 5 mg L^{-1} of BAP + 0.5 mg L^{-1} of IBA and the one added with 2.5 mg L^{-1} of KIN + 0.25 mg L^{-1} of IBA were statistically equal, surpassing the rest of the treatments evaluated in *Nb*, registering an *Nb* of up to 9.25 shoots per explant; however, with the second treatment, larger shoots are obtained, with an *Ab* of 6.73 mm, which facilitates a better manipulation of these when performing the subculture (Table 2).

These results show that the cytokinin:auxin interaction in a 10:1 ratio influences the morphogenesis of the explant of this species, regulating the cell division and growth of the axillary buds for the induction of shoots (Figure 3).

Table 2. Cytokinin-auxin ratio in the activation of axillary buds in the *in vitro* multiplication stage of the ornamental biznaga 'inverted cone of Viereck' (*Turbinicarpus viereckii* subsp. major (Glass & R. A. Foster) Glass).

	Treatment	No. of shoots (*Nb*)	Shoot height (*Ab*) (mm)
1	2.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA	7.57 d	5.04 d
2	5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	7.4 d	4.63 ef
3	7.5 mg L^{-1} BAP + 0.75 mg L^{-1} NAA	5.74 f	4.95 e
4	10 mg L^{-1} BAP + 1 mg L^{-1} NAA	7.57 d	5.3 cd
5	2.5 mg L^{-1} BAP + 0.25 mg L^{-1} IBA	8.85 b	6.8 a
6	5 mg L^{-1} BAP + 0.5 mg L^{-1} IBA	9.24 a	6.07 b
7	7.5 mg L^{-1} BAP + 0.75 mg L^{-1} IBA	8.3 c	4.55 f
8	10 mg L^{-1} BAP + 1 mg L^{-1} IBA	8.36 c	4.54 f
9	2.5 mg L^{-1} KIN + 0.25 mg L^{-1} NAA	8.87 ab	4.54 f
10	5 mg L^{-1} KIN + 0.5 mg L^{-1} NAA	7.54 d	5.07 d
11	7.5 mg L^{-1} KIN + 0.75 mg L^{-1} NAA	5.89 f	4.06 g
12	10 mg L^{-1} KIN + 1 mg L^{-1} NAA	6.5 e	5.24 d
13	2.5 mg L^{-1} KIN + 0.25 mg L^{-1} IBA	9.25 a	6.73 a
14	5 mg L^{-1} KIN + 0.5 mg L^{-1} IBA	9 ab	5.95 c
15	7.5 mg L^{-1} KIN + 0.75 mg L^{-1} IBA	5.21 g	5.01 d
16	10 mg L^{-1} KIN + 1 mg L^{-1} IBA	6.42 e	4.71 ef
	r^2	0.76	0.69
	CV	18.36	26.1
	STD	2.85	2.9
	x	7.61	5.07

Averages with the same letter in each column are statistically equal (Tukey $p \leq 0.05$).

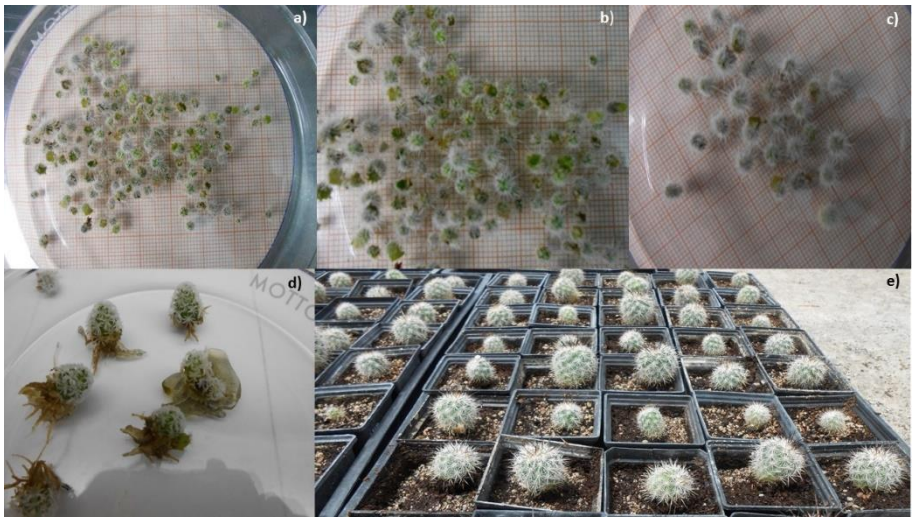

Figure 3. Multiplication of the ornamental biznaga 'inverted cone of Viereck' (*Turbinicarpus viereckii* subsp. major (Glass & R. A. Foster) Glass). a, b c) induction of shoots; d) vitroplants; and e) acclimatized plants.

The KIN:IBA interaction is similar to that reported by Finti *et al*. (2012); Pacheco (2015); Velázquez and Soltero (2001); Villavicencio *et al*. (2020), who found that the cytokinin source has significant effects on the production of shoots in the biznaga 'swallow nest' *Epithelantha micromeris* var. *micromeris* (Engelm.) F. A. C. Weber ex Britton & Rose, cactus pear *Opuntia ficus-indica* (L.) Mill. and 'sacasil' *Echinocereus poselgeri* Lem., so this aspect must be considered in the micropropagation of cacti, since the efficiency of the treatment for the induction of shoots, such as the budding coefficient, are important in the *in vitro* multiplication stage to achieve the final results in the micropropagation, because the number of explants that are established, number of shoots that are generated and vitroplants that are produced will depend on this stage.

As the concentration in the BAP:IBA and KIN:IBA interactions increases, the multiplication rate decreases. The lowest morphogenetic response in the different concentrations evaluated was obtained with the BAP:NAA and KIN:NAA interactions.

This response indicates that naphthaleneacetic acid (NAA) is an auxin that poorly stimulated growth in the axillary buds of *T. viereckii* subsp. major. In other species, such as *Mimosa pudica* (Bianchetti *et al*., 2017), the BAP:NAA association also registered low shoot induction, as the effect reported in the present research.

In cacti such as *Turbinicarpus pseudomacrochele* and *Strombocactus disciformis*, Soltero and Portillo (2015) report that the KIN:NAA interaction can present a significant effect when applied at a concentration of 3 to 10 mg L^{-1} of KIN, combined with a low concentration of 0.02 mg L^{-1} of NAA, so it is necessary to consider these factors in this process, since the morphogenetic response of the explant will depend significantly on this interaction and the rest of the components of the culture medium.

Conclusions

The regeneration of this species in risk status, as well as its *ex-situ* conservation can be carried out with *in vitro* multiplication, where the cytokinin-auxin ratio influences the morphogenetic capacity and stability of the explant, from which the proliferation of axillary shoots can be induced.

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