Colegio de Postgraduados

# Mass propagation of tobala mezcal maguey (*Agave potatorum* Zucc.) in a temporary immersion system compared with a solid medium

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**ABSTRACT**

**Objective**: To assess a temporary immersion system for the *in vitro* propagation of *Agave potatorum* Zucc., compared with the traditional micropropagation technique that uses a solid medium.

**Design/Methodology/Approach**: The effect of treatments in a solid medium with low and high doses of the BA (Benzylaminopurine) growth regulator (0.5 mg $L^{-1}$ and 2 mg $L^{-1}$) on the number of sprouts per explant was assessed in a first phase. Since the best treatment was 2 mg $L^{-1}$ of BA, three forms of propagation were considered: solid medium, liquid medium in a paper bridge, and liquid medium in a temporary immersion system.

**Results**: From the initial test, an average of 6.6 shoots per explant were obtained with 2 mg $L^{-1}$ of BA. Regarding the different systems, the solid medium, the paper bridge, and the temporary immersion system recorded 6.4, 7.2, and 14.4 shoots per explant, respectively.

**Findings/Conclusions**: Mass sprout production is higher in the temporary immersion system, as a consequence of the use of a liquid medium that increases the absorption of nutrients and regulators, combined with the injection of air with oxygen that can accelerate cellular processes.

**Keywords**: Micropropagation, RITA®, liquid medium.

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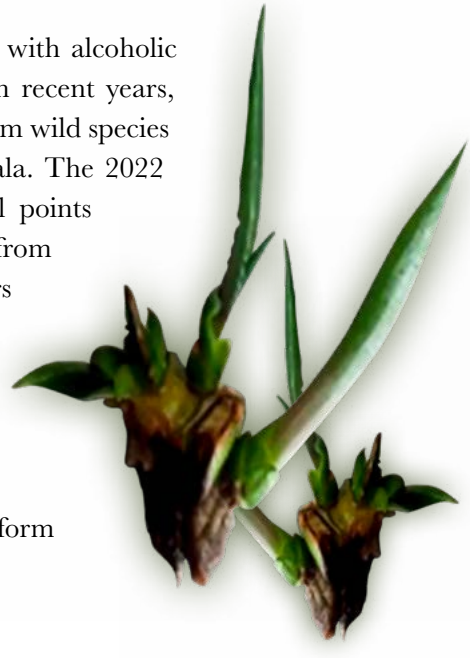
## INTRODUCTION

Maguey or agave (*Agave* L.) production is associated with alcoholic beverages such as pulque, sotol, tequila, and mezcal. In recent years, mezcal demand has grown exponentially, particularly from wild species such as *Agave potatorum* Zucc., popularly known as tobala. The 2022 statistical summary of the Mezcal Regulatory Council points out that the production of certified mezcal increased from 1,044,696 million liters (2012) to 8,099,591 million liters (2022), an indirect indication of the plant demand required to satisfy mezcal production. In the case of *A. potatorum*, it does not generate shoots (adventitious sprouts) and its propagation is therefore asexual (Gentry, 2004). Consequently, several authors have explored propagation per tissue culture as an alternative form



of multiplication which does not require waiting for seed production. Bello *et al*. (2023) recently reported the use of temporary immersion systems for the *in vitro* propagation of this species. Previous research has raised interest on the assessment of the difference in the multiplication rate of the traditional micropropagation technique in a solid medium versus a temporary immersion system.

## METHODOLOGY

### Establishment under aseptic conditions

To set up the initial material of *A. potatorum* under aseptic conditions, basal buds were taken from young 8-month-old plants. The collection was carried out in 2 locations (Table 1). All specimens were disinfected as follows: 4 washes with liquid soap and rinsing with running water, immersion in a 70% alcohol solution for 2 min, followed by immersion in a 30% sodium hypochlorite solution for 18 min, and finally 3 rinses with sterile water (in a laminar flow hood). At the end of this process, the explants in test tubes were randomly assigned to the media under evaluation.

### Culture media

The study was carried out in 2 sequential tests. Three treatments were assessed in the first test, using the Murashigue and Skoog (1962) "MS" solid medium (with vitamins) as basis. Treatments consisted of the application of different doses of the BA (Benzylaminopurine) growth regulator: treatment 1, 0 mg $L^{-1}$ (control); treatment 2, 0.5 mg $L^{-1}$ (low dose); and treatment 3, 2.0 mg $L^{-1}$ (high dose). The growth regulator had three levels, which matched the doses in a solid medium.

In the second test, the high dose (2.0 mg $L^{-1}$) of growth regulators in solid medium —which had the best biostatistics performance in the first test— was considered as a reference value; two immersion systems were also analyzed as part of this test. Afterwards, the immersion systems factor —keeping 2.0 mg $L^{-1}$ of BA as a constant— included 3 treatments: a) Treatment 1, solid medium as control; b) Treatment 2, liquid medium in paper bridge (filter paper as bridge and 7 mL of medium per tube); and c) Treatment 3, liquid medium in a RITA® immersion system (50 mL of medium in the container, immersion every 4 h, and 3-min immersion periods).

In both the preliminary test and the immersion system comparison, the final measurement of the number of shoots variable and the length per explant variable was made at 60 days. In both tests, the experimental units (test tubes) were randomly assigned to the treatments. In the conditions under which the study was carried out, a completely randomized design was considered, in which the growth regulator dosage and the immersion system were the factors in the first and second tests, respectively.

**Table 1**. Origin of shoots used as initial explants.

| Accession | Sprout (Number) | Common name | Scuentific Name | State | Location |
|---|---|---|---|---|---|
| A-O-01 | 20 | Agave Tobalá | *Agave potatorum* | Oaxaca | Miahuatlán Cerro Metate |
| A-O-02 | 30 | Agave Tobalá | *Agave potatorum* | Oaxaca | San Pedro Teozacoalco<br>San José Rio Minas |

**Table 2**. Treatments in solid medium to determine sprout formation response (Phase 1).

| Treatment | Media | Growth regulator and concetration |
|---|---|---|
| 1 | Murashigue y Skoog | Sin regulador (testigo) |
| 2 | Murashigue y Skoog | BA 0.5 mg $L^{-1}$ (dosis baja) |
| 3 | Murashigue y Skoog | BA 2.0 mg $L^{-1}$ (dosis alta) |

**Table 3**. Treatments of the comparison of *in vitro* propagation systems (Phase 2).

| Treatmet | Sistem | Media | Growth regulator |
|---|---|---|---|
| MSBALFS | Medio sólido | MS | BA 2 mg $L^{-1}$ |
| MSBALFP | Puente de papel | MS | BA 2 mg $L^{-1}$ |
| MSBALSI | RITA® | MS | BA 2 mg $L^{-1}$ |

**Plant acclimatization**

As suggested by Monja-Mio *et al.* (2020), plant acclimatization was carried out in two stages: laboratory and greenhouse. In both cases, the same substrate was used: 3 equal parts of Peat Moss®, vermiculite, and perlite sterilized in an autoclave at 120 °C for 30 min. For laboratory acclimatization, the plants remained in plastic containers with lids for 6 weeks. Once they were taken to the greenhouse, the plants were transplanted to 1-L pots with the same substrate for 60 days, before they were transferred directly to soil of the field. After 60 days, their development was assessed.

**Statistical analysis**

**Growth regulator dosage test**

Based on the experimental design, the data were analyzed considering a Generalized Linear Model which, according to Stroup (2014), has the following linear predictor:

$$\eta_j = \eta + \tau_j \text{ y } \eta_j = \log\left(\mu_j\right), \text{ then, } \mu_j = \exp\left(\eta + \tau_j\right).$$

Where: $y_{ij} \sim Negative\ Binomial\left(\mu_j, \mu_j + \psi\mu_j^2\right)$ is the number of sprouts in the *i-th* explant of the *j-th* treatment. In this case, the variance contains an overdispersion term $\psi\mu_j^2$: the larger $\psi$, the larger the overdispersion. $\eta$ represents the mean value for the reference level. $\tau_j$ represents the log-mean difference for the level of the treatment with the reference level.Additionally, $y_{ij} \sim Gamma\left(\mu_j, \Phi\mu_j^2\right)$ is the length of sprouts in the *i-th* explant of the *j-th* treatment. Since the experimental units did not form clusters, neither intensively, nor naturally, the were considered independent, within and between treatments.

**Immersion systems test**

In this case, the number of sprouts variable took into consideration the negative binomial distribution, while the length of sprouts variable was modeled using the inverse Gaussian distribution. Therefore, $y_{ij} \sim IGAUSS\left(\mu_j, \Phi\mu_j^3\right)$.

Given the nature of the statistical model —which identifies the stochastic process of data—, the analysis was performed using PROC GLMMIX (SAS, 2018), specifying the negative binomial distribution (for the number of sprouts) or the range of the Gaussian inverse (for their length).

## RESULTS AND DISCUSSION

### Growth regulator dosage test, Phase 1

#### Number of sprouts

The basic statistical values for this variable were: minimum=1, mean=3.63, maximum=11, and variance=9.4. The variance registered an almost triple value. Figure 1 shows that the number of sprouts of treatment 1 recorded the lowest value, while treatment 3 was slightly higher than the mean of 6 (empty circle). The variability of the observations in treatment 3 was also significantly higher than in treatments 1 and 2. The interquartile range of treatment 3 was 6.

The statistical analysis that considered the negative binomial distribution helped to identify the differential effect on the number of sprouts variable of the treatments assessed. Therefore, F had a value of 17.31 (p-value <0.0001), based on 2 and 27 degrees of freedom in the numerator and in the denominator, respectively. Figure 2 shows the means predicted for each treatment, as well as the 95% confidence intervals. Once again, these results confirm that treatment 3 had a better mean value (6.6) than treatments 2 and 3. The analysis of the multiple comparison of pairs of treatments using Tukey's test reported that 3 perfectly identifiable independent groups are formed (Table 3).

The statistical analysis based on the negative binomial distribution was considered appropriate, because the Shapiro-Wilks test for normality of the residuals showed a value of 0.94 and a p-value of 0.1176. In fact, compared with those predicted under normal conditions, the observed percentiles of the Pearson residuals practically fall in a straight line that passes through the y-intercept (Figure 3).

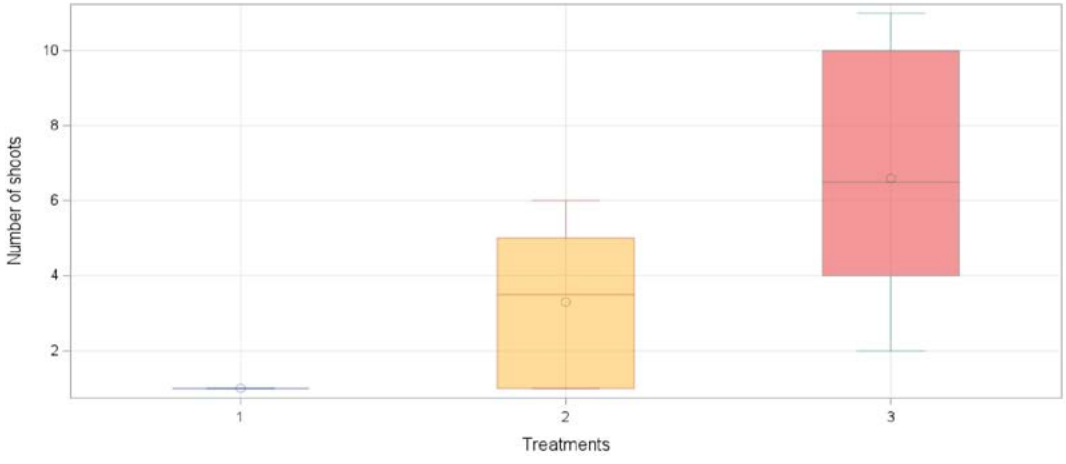



**Figure 1**. Distribution of the number of shoots per treatment.

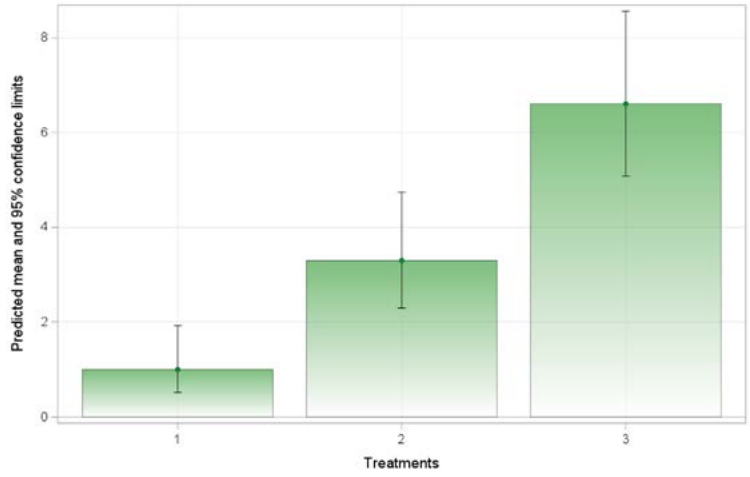



**Figure 2**. Mean number of shoots (prediction) and 95% confidence intervals.

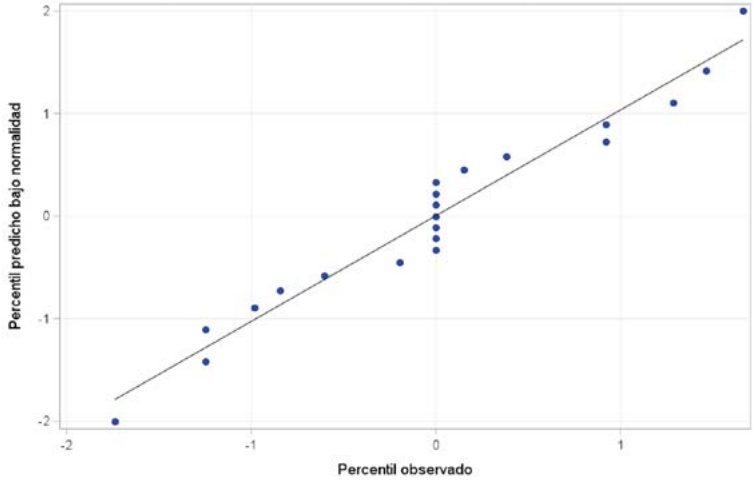



**Figure 3**. Percentiles (observed and modeled under normality) of the residuals resulting from the analysis of the negative binomial distribution.

**Table 4**. Tukey's grouping considering differences in predicted means.

| Treatment | Media | Cluster |
|---|---|---|
| 3 | 6.6 | A |
| 2 | 3.3 | B |
| 1 | 1.0 | C |

## Shoots length (cm)

Regarding sprout length, treatment 3 had the highest average, slightly exceeding 4.5 (empty circle). Treatment 1 was the most variable with an interquartile range of 1 (Figure 4).

The statistical analysis that considered the sprout length variable with gamma distribution detected that this variable behaves differently in each of the treatments. F had a value of 7.85 (p-value=0.0021), calculated starting from 2 degrees of freedom in

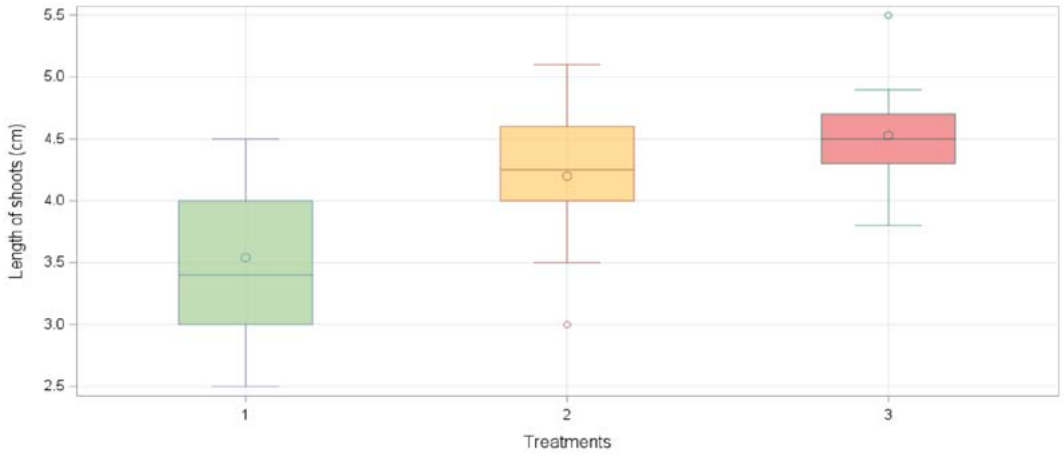



**Figure 4**. Sprout length per treatment.

the numerator and 27 in the denominator. In light of these results, at least 2 means are different. Figure 5 shows the predicted means and 95% confidence intervals for the sprout length variable for each treatment. Treatments 1 and 2 were surpassed by treatment 3. According to Table 5, two groups were analyzed through the multiple comparison of pairs of treatments using Tukey's test (0.05 significance level); treatments 2 and 3 were included in the same group —a logical conclusion of the observation of an overlap in a large percentage of their respective confidence intervals.

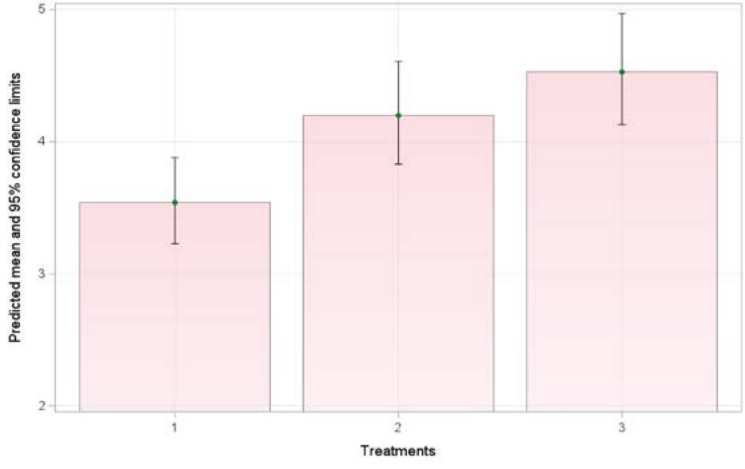



**Figure 5**. Predicted mean of sprout length and 95% confidence intervals.

**Table 5**. Tukey grouping considering differences of the predicted means (alpha=0.05).

| Treatment | Media | Cluster |
|---|---|---|
| 3 | 4.53 | A |
| 2 | 4.20 | A |
| 1 | 3.54 | B |

The Shapiro-Wilks test for normality of the residuals obtained a value of 0.97 and a p-value of 0.6620. The percentiles observed for the Pearson residuals versus those predicted under normality generally fall on a straight line crossing the y-intercept. Consequently, a gamma distribution-based statistical analysis is considered appropriate.

**Propagation systems test, Phase 2**

**Number of shoots**

For this variable, the minimum, mean, maximum, and variance values were 3, 9.4, 18, and 16.73, respectively. Variance was almost two times higher than the mean. Therefore; this result (as in the previous test) justified the use of the negative binomial distribution. Results showed that treatment 3 (RITA® temporary immersion system) generated 14.4 sprouts per explant, while treatment 1 (traditional system with a solid medium) barely reached 6.4 sprouts per explant and treatment 2 (immersion in paper bridge) recorded just 7.4. It should be noted that treatment 1 had the greatest variability, leading to a variance of 5.3 and an interquartile range of 4 (Figure 7).

Considering the F-test value —equal to 19.30, based on 2 and 27 degrees of freedom in the numerator and the denominator, respectively—, as well as a significance level of 0.05, the treatments have a significant differential effect on the discrete number of the sprouts variable. Consequently, the means predicted for treatments 1, 2, and 3 (6.4, 7.4, and 14.34, respectively), as well as the 95% confidence intervals, show that treatment 3 significantly exceeded the other two treatments under study (Figure 8). In fact, pairwise multiple comparisons of observations made with Tukey's test identified 2 groups (Table 6). It should be noted that treatments 1 and 2 were put in a single group (Table 7).

Statistical analysis based on negative binomial distribution is considered appropriate, because the Shapiro-Wilks test for normality of Pearson residuals yielded a statistic of 0.97 and a p-value of 0.7122. Furthermore, the graph of the percentiles observed in Pearson

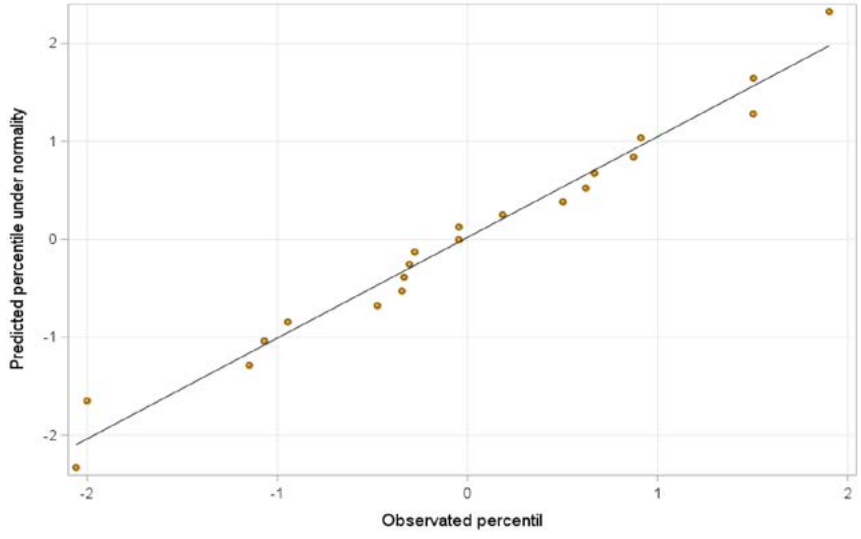



**Figure 6**. Percentiles (observed and modeled under normality) of Pearson residuals obtained through gamma distribution.

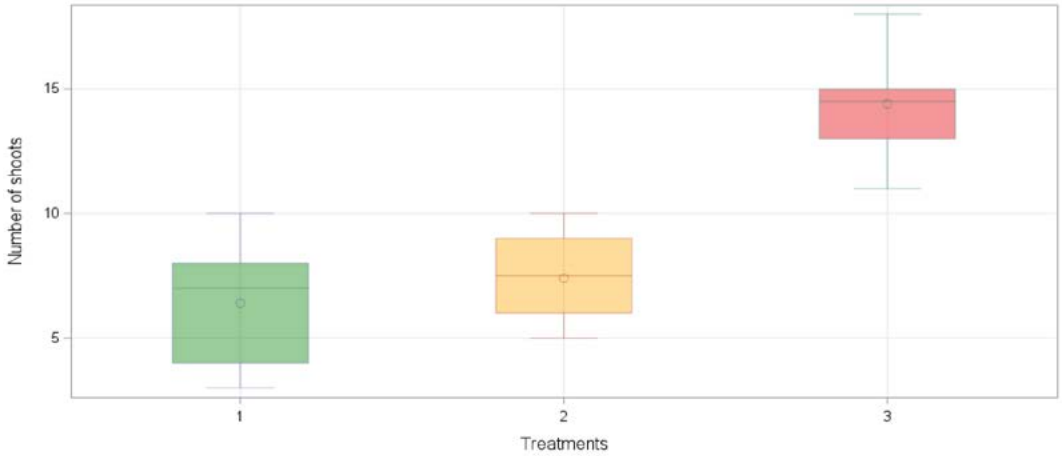



**Figure 7**. Number of sprouts per treatment.

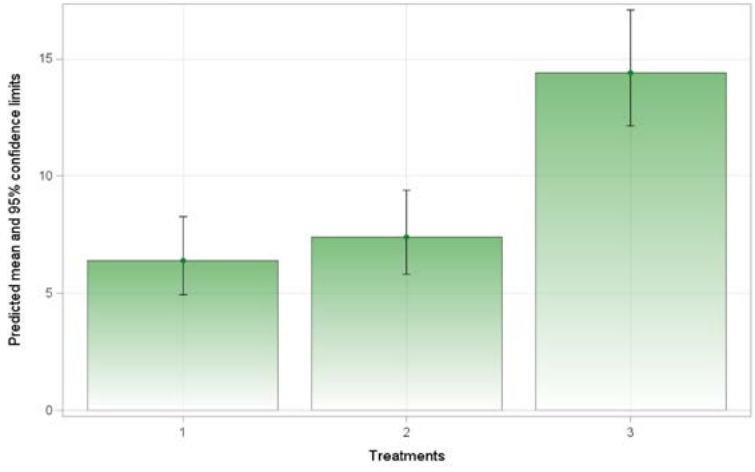



**Figure 8**. Predicted mean and 95% confidence limits of number shoots of diferents treatments.

**Table 6**. Tukey grouping considering differences between predicted means (alpha=0.05).

| Treatment | Media | Cluster |
|---|---|---|
| 3 | 14.40 | A |
| 2 | 7.40 | B |
| 1 | 6.40 | B |

residuals and those modeled considering normality indicates that they are distributed along a straight line that passes through the y-intercept (Figure 9).

## Shoots length

This variable recorded values of 2, 3.42, 6.90, and 2.45, for the minimum, mean, maximum and variance, respectively. Treatment 3 reached a mean of 5.49 cm; this result

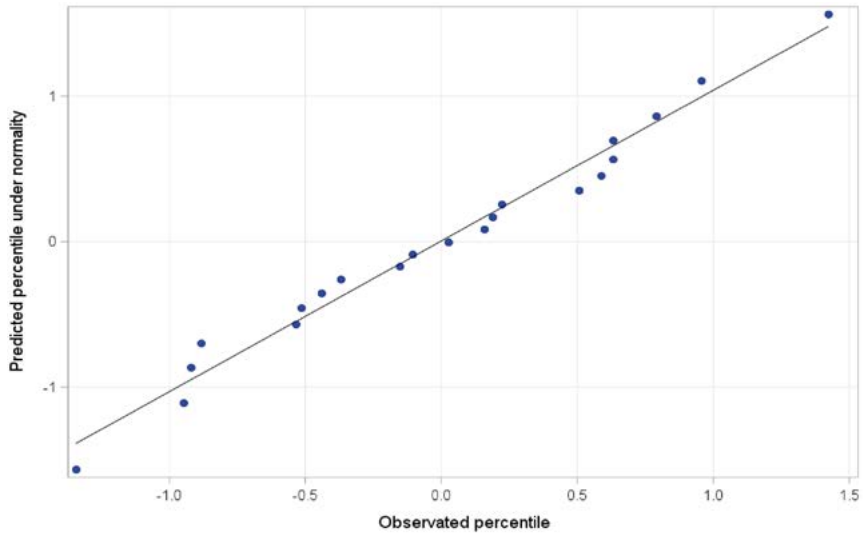



**Figure 9**. Percentiles (observed and modeled under normality) of Pearson residuals obtained through negative binomial distribution.

was significantly higher than treatments 1 and 2, which showed similar mean values (≤2.5 cm). Treatment 3 also showed a slightly larger interquartile range than treatment 2 (Figure 10).

The value of the F-test was 87.61, with a p-value significantly lower than 0.05, suggesting that the immersion systems have a different influence on the behavior of the sprout length variable. The mean of treatment 3, as expected, exceeded the means of treatments 1 and 2 by a little more than twice. In this sense, the 95% confidence interval of treatment 3 had noticeably different values than the other 2 treatments (Figure 11). Therefore, the analysis of the multiple pairwise comparisons of the means made with Tukey's test generated one group made of two treatments (1 and 2) and a group with a single treatment (3) (Table 7).

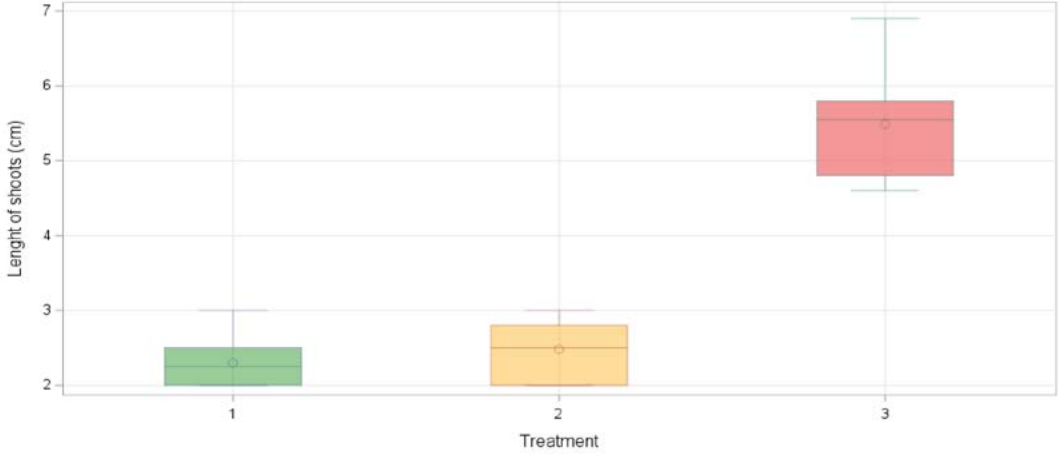



**Figure 10**. Shoots length per treatment.

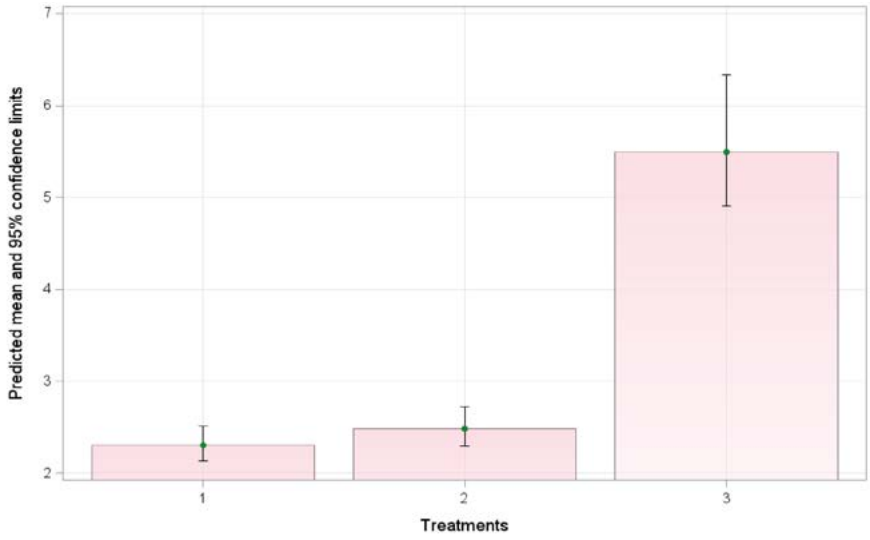



**Figure 11**. Predicted mean and confidence intervals of sprout length from different treatments of the immersion system.

**Table 7**. Tukey grouping considering differences between the predicted means (alpha=0.05) for the sprout length variable.

| Treatment | Media | Cluster |
|---|---|---|
| 1 | 2.30 | A |
| 2 | 2.48 | A |
| 3 | 5.49 | B |

Even though dispersion of the percentiles of residuals observed and modeled under normality deviate slightly from the y-intercept, modeling of the sprout length variable in the immersion system can be considered acceptable, given the value (W=0.95) and p-value (0.2190) yielded by the Shapiro-Wilks test of normality (Figure 12).

Meanwhile, the number of sprouts was significantly higher with the temporary immersion system (14.4) than with the solid medium (6.4). Authors such as Ríos-Ramírez *et al.* (2017) recorded a proliferation of 32 sprouts per explant in *A. agustifolia*, on a solid medium with a 4 mg $L^{-1}$ concentration. Monja-Mio *et al.* (2021) found similar results and reported higher sprout production in *A. angustifolia*, when they compared a solid medium with the RITA® temporary immersion system. Authors such as Ramírez-Mozqueda *et al.* (2022) and Correa-Hernandez *et al.* (2022) reported a substantial increase in sprout production using the Ebb-and-Flow temporary immersion system with *Agave potatorum*. Ontaneda *et al.* (2020) mentioned the cost-wise efficiency of the temporary immersion systems, resulting from the increase and obtaining of *in vitro* plants compared with the conventional solid medium system, although they failed to compare it with other temporary immersion systems.

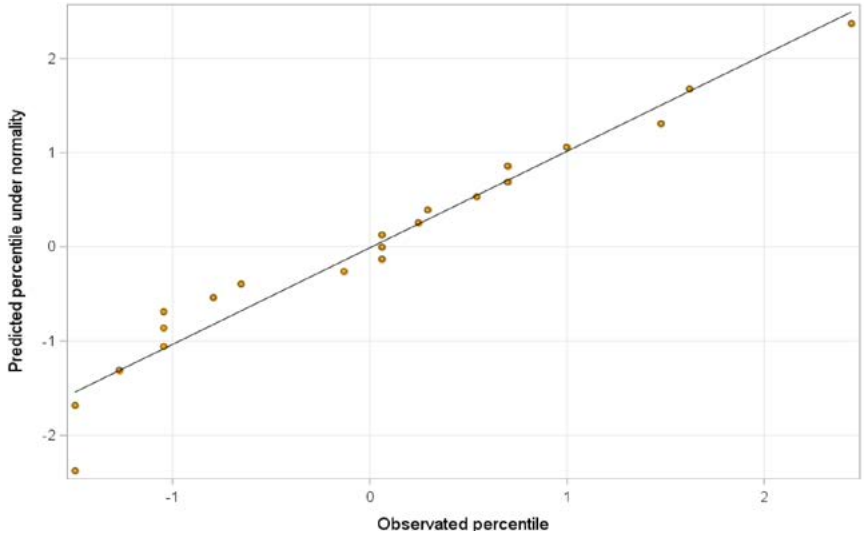



**Figure 12**. Percentiles (observed and modeled under normality) of Pearson residuals obtained by inverse Gaussian distribution.

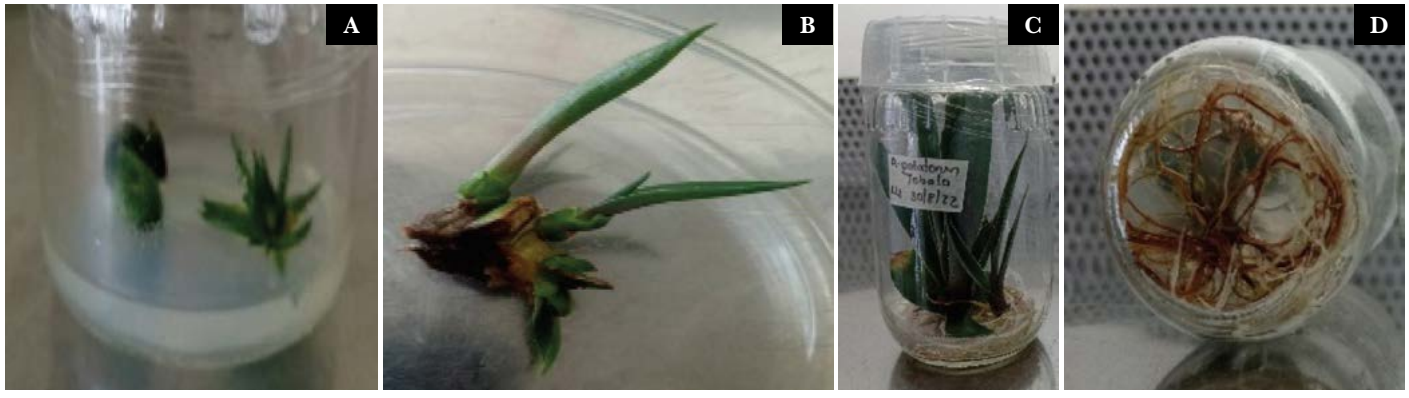



**Figure 13**. Explant responses. A: solid medium B: sprouts grown in an immersion system, C: *in vitro* seedling growth, D: developed roots.

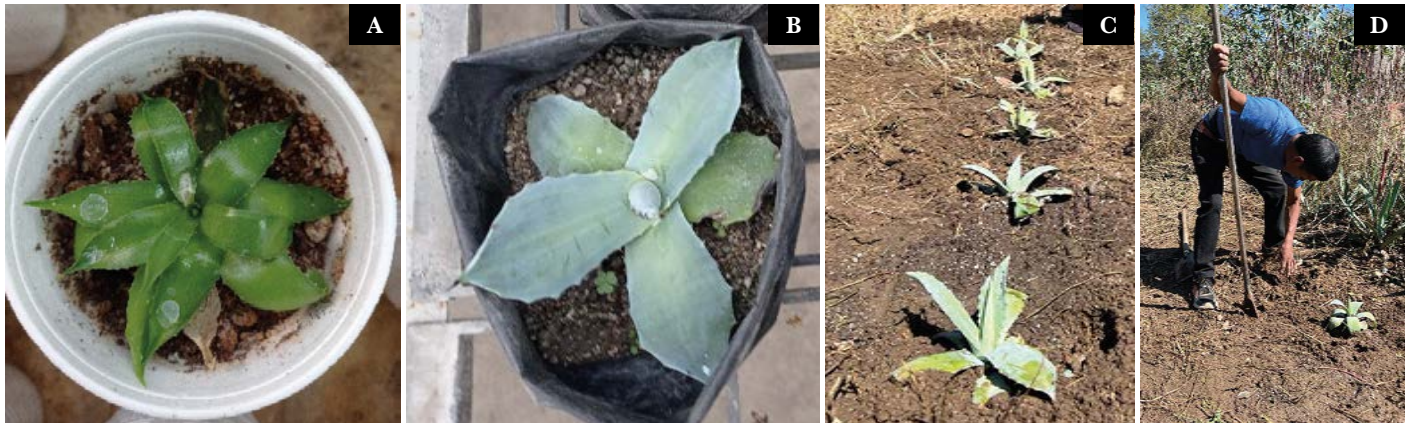



**Figure 14**. A: acclimatization in laboratory. B: hardening off in greenhouse. C and D: field establishment of *Agave potatorum* propagated under *in vitro* conditions.

### Plant acclimatization

The study specimens were initially acclimatized in the growth laboratory chamber (93% survival) and plant hardening off was carried out in the greenhouse. Then, plants were taken to the field in Oaxaca where they were received by a cooperating farmer. One-hundred percent of the plants were finally established after a 60-day assessment.

## CONCLUSIONS

Propagating Tobala agave (*Agave potatorum*) under *in vitro* conditions by means of a temporary immersion system (RITA®) is a more efficient mass propagation technique, both for shoots formation and for growth and development (length), than the traditional solid medium, achieving a higher growth rate of the sprouts per explant which satisfactorily reaches the acclimatization and field establishment phases.

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