Colegio de Postgraduados

Assessment of tepojal as a support medium in *in vitro* germination of *Barkeria whartoniana* (Orchidaceae) and subcultures of *B. uniflora* and *B. scandens*

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ABSTRACT

Objective: We evaluated the use of tepojal as a substitute for bacteriological agar for *in vitro* culture of three species of Mexican orchids of the genus *Barkeria*. We had three objectives: (1) evaluate the use of tepojal for the *in vitro* germination of *B. whartoniana* seeds; (2) compare the growth and survival of *B. uniflora* and *B. scandens* plants in *in vitro* culture on bacteriological agar and tepojal, and (3) compare the early *ex situ* survival of the *B. uniflora* and *B. scandens* previously cultivated *in vitro* on bacteriological agar and tepojal.

Design/Methodology/Approach: For the growth experiment, 1,050 seedlings of *Barkeria scandens* and *B. uniflora* were used. Two types of culture medium were prepared: (1) Liquid with tepojal and 40% MS medium with dextrose, yeast, coconut water, activated carbon and refined sugar, and (2) solid at 40%, with the same elements as the liquid one, but with 6 g of bacteriological agar. For the germination experiment of *Barkeria whartoniana* in tepojal we use seeds from a mature (open) capsule. After disinfection, seeds were sown in tepojal with liquid medium and in a solid (agar) culture using a syringe technique. All the seeds were sown in 100% p668 MS medium with 100 mL/L of coconut water and 15 g/L of refined sugar and using in both the MS medium.

Results: *Barkeria uniflora* seedlings had a greater growth than in *B. scandens* regardless of the type of treatment. When comparing within each species, we found that the two treatments (tepojal *vs.* agar) did not produce differences in the growth of the shoot of both species. Roots growth was influenced by both the effect of the species and the treatment, as the longest roots were found in tepojal medium. Seed germination observed in the liquid medium with tepojal was not apparently different from that in agar. In both cases the entire surface of the jar was covered with green protocorms.

Findings/Conclusions: Tepojal did function well as a substrate to germinate *Barkeria whartoniana*, but tests must continue to be carried out to evaluate its effectiveness. As no differences were found for aerial growth between agar and tepojal in *Barkeria scandens* and *Barkeria uniflora*, we consider this substrate as a good substitute for agar because it can produce more biomass per gram of culture medium used; therefore, if we consider the costs the use of tepojal is much cheaper and much easier to get.

Keywords: Agar substitution, *in vitro* culture, *ex vitro* survival, orchid.

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INTRODUCTION

Many efforts have been made in the last 30 years to find a suitable substitute for bacteriological agar due to its high cost, low accessibility, and high demand (Jain-Raina and Babbar 2011). However, a noneffective substitute has been found (Khoobbakht *et al.*, 2024), particularly as a support medium for *in vitro* orchid cultivation. Bacteriological agar is a material that is not easy to find in the market and expensive for small producers (McGuffey *et al.*, 2018), for which there is a continuous search for alternatives to replace it (Gordo *et al.*, 2012; Herrera-Toledo *et al.*, 2013; Sánchez-Cardozo, 2019).

Agar is mainly extracted from marine Rhodophyta; however, populations of these wild algae are vulnerable due to massive extraction (Porse and Rudolph, 2017). Agar is composed by agarose and agaropectin, and it is highly prized because it remains gelled at room temperature and it has high clarity and is harmless to organisms being cultivated (Jain and Babbar, 2005). Some commercial products, such as Phytagel, Gelrite and Natugel, have been used as substitutes for agar. Nevertheless, these fail to equalize the firmness that bacteriological agar achieves. Also, plants being cultivated in them have greater oxidation problems and higher mortality. Other substances with gelling properties such as starches, gums, and polymers, both from plants and bacteria have also been tested (Ozel *et al.*, 2008; Gordo *et al.*, 2012). These substances have reduced production costs of *in vitro* culture media up to 70% but fail to reproduce the same results as agar (Fujiwara *et al.*, 1993).

Because suitable gelling agents have not been found, some culture media made with materials that make a solid structure, but which have the liquid nutrient medium, have been tested. For example, culture media have been made with coconut, cotton and betel nut fibers, starches like sweet potato, corn and potato, polyurethane foam, chopped litter leaves, isubgol, some gums such as guar, gellan, katira and cassava flour have shown good results (Moraes-Cerdeira *et al.*, 1995; Afreen-Zobayed *et al.*, 2000; Jeyaseeli and Raj, 2010). For orchids, some of these substrates have been used for in vitro germination for some genera such as *Cymbium*, *Cattleya* and *Stanhopea* (Deb and Pongener, 2010; Aggarwal and Nirmala, 2012). Recently, tezontle has been used together with coconut fiber in *in vitro* cultivation of orchids (Flores-Hernández *et al.*, 2017).

Tepojal is a vitreous extrusive volcanic rock. It is a light inert mineral, with a neutral pH. In agriculture it is widely used in the preparation of substrates for various crops since it facilitates aeration and produces light substrates. Tepojal, as an inert rock, does not generate changes in the chemical composition of the culture medium and it does not weather easily either with heat or with water. This is important, as it remains intact during sterilization procedures (even autoclaved) and during the time it is used as an *in vitro* culture medium.

In this study, we evaluated the use of tepojal as a substitute for bacteriological agar for *in vitro* culture of three species of orchids of the genus *Barkeria*. We had three objectives: (1) evaluate the use of tepojal for the *in vitro* germination of *B. whartoniana* seeds; (2) compare the growth and survival of *B. uniflora* and *B. scandens* plants in *in vitro* culture on bacteriological agar and tepojal, and (3) compare the early *ex situ* survival of the *B. uniflora* and *B. scandens* previously cultivated *in vitro* on bacteriological agar and tepojal. We expected that, due to the properties of tepojal, there would be no differences between the cultures of both species in tepojal and bacteriological agar.

METHODS

Study species

The genus *Barkeria* has 16 species of which 12 are endemic to Mexico. It is distributed along the Pacific coast and their main habitat is deciduous forests (Soto-Arenas, 2005). Of the 16 species of the genus, seven are within a category of NOM-059, among them *B. scandens* and *B. whartoniana* are in special protection. This genus is composed by herbs with thickened stems or small pseudobulbs, they have thick roots that tend to form dense masses,

they are epiphytes or lithophytes with cespitose or scandent growth. Their inflorescence is terminal, flowers are 2 to 5 cm in size, and their leaves are deciduous.

Selection and sterilization of tepojal

Tepojal was purchased at a gardening store in the Madreselva market in Xochimilco, Mexico City. For subcultivation in tepojal, particles the size of 2 mm in diameter was used, while for the germination experiment in fine tepojal, smaller particles were used. The 2 mm thick tepojal was washed in running water until the water was clear and dried by placing it under direct sunlight in porous, plastic trays. Once dry, the tepojal was placed in 90 g glass jars with plastic screw caps and 300 ml of distilled water. The jars with tepojal were autoclaved two times (one with only tepojal and other with liquid culture medium) (Felisa) at 120 °C and a pressure of 1.5 kg/cm^2 for 20 min. After that, they were sealed with self-adhesive plastic and stored on shelve.

Preparation of solid culture medium for subcultures

To make a solid culture medium, a commercial preparation of Murashige and Skoog medium (M 519) at 40%, 1 g/L of dextrose, 1 g/L of yeast, 100 ml/L of coconut water, 2 gr/L of activated carbon, 15 gr/L of refined sugar was used. The medium was prepared on a shaker rack in beakers using distilled water. At the end of this process the pH was adjusted to 5.6±0.2, either with HCL (hydrochloric acid) or NaOH (sodium hydroxide) both at 1 N. Once the culture medium was prepared and the pH adjusted, the solution was heated to the boiling point and 6 g of bacteriological agar were added little by little until it was completely diluted. 50 mL of this culture medium were immediately poured into 90 g glass bottles with plastic screw caps. The vials with the culture medium were sterilized the same way as tepojal. After that, jars were cooled outdoors, shaking them from time to time so that the carbon remained uniformly suspended in the medium. Once the culture media had gelled, the glass bottles were sealed with plastic wrap and stored until use (less than a week).

Liquid medium with tepojal for subcultures

The liquid culture medium was prepared in the same way as the solid culture medium, except that it was not brought to a boil because bacteriological agar was not added. In 90 g jars with 75 g of sterilized 2 mm diameter tepojal, 35 ml of the liquid medium and sterilized in an autoclave at 120 °C and a pressure of 1.5 kg/cm^2 for 17 min. During the pouring process, the culture medium was stirred to keep the activated carbon suspended. Once the vials came out of the autoclave, they were sealed with plastic wrap and stored.

Vegetative material

Seeds from *Barkeria scandens*, *B. uniflora* and *B. whartoniana* were obtained from a mature capsule cultivated in the "Miguel Ángel Soto Arenas" orchidarium at the Faculty of Sciences, UNAM. Propagules from *Barkeria scandens* and *B. uniflora* were germinated in 40% solid MS medium and allowed to grow for 6 months before the experiment. For *B. whartonianan* seeds were stored for two months until the germination experiment. A

total of 1,050 seedlings of *Barkeria scandens* and *Barkeria uniflora* were used for the growth experiment.

Seed disinfection

Disinfection was performed with a 15% (v/v) sodium hypochlorite solution (domestic bleach) for 15 minutes, stirring the seeds constantly under aseptic conditions in a laminar flow hood, 3 rinses were performed with sterile distilled water.

Germination of *B. whartoniana* seeds

After disinfection, seeds were sown in liquid and solid culture using a syringe technique. 1 ml of the seed solution was placed in each vial. All the seeds were sown in 100% p668 MS medium with 100 mL/L of coconut water and 15 g/L of refined sugar. 6 g of agar were used, and 30 ml of medium were occupied in each bottle for the solid culture medium. For the liquid culture medium with tepojal, 25 mL of the nutrient solution and 15 g of fine-grained tepojal were used. The seeds were incubated at 25 °C, under a photoperiod of 16 h light/8 h dark.

Growth experiment

Two types of culture medium were prepared to evaluate the use of tepojal as a substitute for bacteriological agar in the development (growth and survival) of *Barkeria scandens* and *B. uniflora* seedlings. The first was liquid with tepojal and 40% MS medium with dextrose, yeast, coconut water, activated carbon and refined sugar. The second was solid at 40%, with the same elements as the liquid one, but with 6 g of bacteriological agar. Seedlings of both species were planted in solid culture medium (with agar) and in liquid culture medium with tepojal as a substrate. In total, 15 randomly selected plants were taken from a previous culture in solid MS medium. At the beginning of the experiment 140 seedlings were randomly selected inside the laminar flow hood and measured using a laminated millimeter paper (previously superficially sterilized with 70% alcohol). The seedlings were measured at the beginning and at the end of the experiment, taking two growth measures: (1) length of the shoot from the base of the stem to the apex of the longest leaf and (2) the length of the root from the base of the stem to the apex of the longest root. Once the six months of *in vitro* culture had elapsed, the plants were taken out for their *ex-vitro* culture and sown in small baskets (1 inch in diameter) in a mixture of tepojal and bark (2:1), and after six months their survival *ex vitro* was evaluated.

Statistical analysis

For growth of the aerial part of the plant and root growth, we fitted a set of linear models that included the initial size of the plant, the species, and the treatment in which the seedlings were found (agar or tepojal) as independent variables. The dependent variables were log transformed to be normalized. In total, 15 different nested models were obtained, which included all possible combinations with the variables, as well as the interactions between them and a null model where no variable has an effect. For probability of survival, 5 different nested linear mixed effects models were fitted. We converted the response

variable with the arc sine of the square root to normalize it. These models included the treatment, the species identity, and their interaction as well as a null model. The jar used for germination of each seed was included as a random effect. All the analyzes were performed in R (R Core Team 2018) and the mixed effects models of fitted using the statistical package "lme4" (Bates *et al.*, 2015). Model with the lowest Akaike information criteria (AIC) value was accepted as the best model, and models that did not differ by more than two units (Δ AIC) from the best model were considered equally acceptable.

RESULTS AND DISCUSSION

Evaluation of the longitudinal growth of *Barkeria uniflora* and *B. scandens*

Barkeria uniflora seedlings shoots were bigger than in *B. scandens* regardless of the type of treatment. When comparing within each species, we found that the two treatments did not produce differences in the shoot growth (Figure 1). According to the fitted linear models, the best model included the initial size and the species as factors that influence the final size of the shoot (Table 1). It is important to note that the next best model did not differ by more than two AIC units, as was the case for the rest of the models in the set. Thus, the treatment may be slightly affecting the final size of the aerial part of seedlings, particularly for *B. uniflora*.

Root growth

Final root growth was influenced by both the species and the treatment (Table 2). Longest roots were found for *Barkeria uniflora* in the tepojal treatment, followed by those of *B. scandens* also in tepojal, while the smallest were those of *B. scandens* in agar (Figure 2). The best model found included all the independent variables without their interactions (Table 2). This means that the initial size of the seedlings, the species, and the treatment in which they were found determined the growth of the roots. It should be noted that there are four other linear models that could not be ruled out because their AIC did not differ in more than two units (Table 2). In general, these four models that had a good fit included

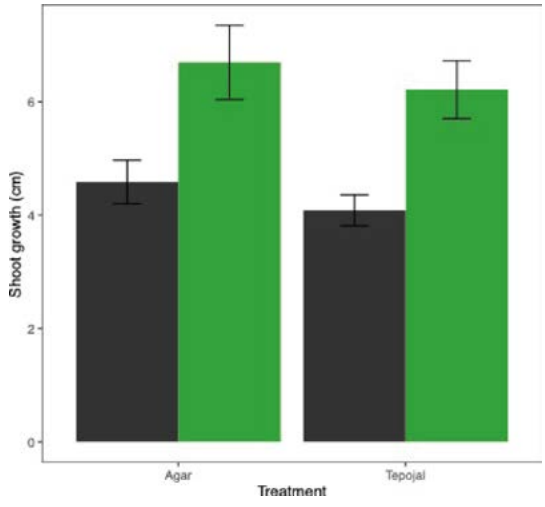

Figure 1. Shoot growth of *Barkeria uniflora* (green) y *B. scandens* (gray) cultivated *in vitro*.

Table 1. Linear mixed effect models used to evaluate shoot growth.

Shoot growth models	df	AIC	Δ AIC
Initial size+Species	4	133.68	0
Initial size+Species+Treatment	5	134.17	0.49
Initial size+Treatment+Species+Initial size×Treatment	6	135.83	2.14
Initial size+Treatment+Species+Initial size×Species	6	136.16	2.47
Initial size+Treatment+Species+Species×Treatment	6	136.17	2.48
Initial size+Treatment+Species+Species×Treatment+Initial size×Treatment	7	137.80	4.11
Initial size+Treatment+Species+Initial size×Species+Initial size×Treatment	7	137.83	4.14
Initial size+Treatment+Species+Initial size×Species+Initial size×Treatment	7	137.83	4.14
Initial size+Treatment+Species+Species×Treatment+Initial size×Species	7	138.15	4.46
Initial size+Treatment+Species+Species×Treatment+Initial size×Species+Initial size×Treatment	8	139.80	6.11
Initial size	3	142.24	8.55
Initial size+Species	4	143.24	9.55
Species	3	151.69	18.00
Treatment+Species	4	153.49	19.80
Null	2	164.98	31.29
Treatment	3	166.97	33.28

df=degrees of freedom, AIC=Akaike information criteria, Δ AIC=difference in AIC between each model and the best one.

Table 2. Linear mixed effect models used to evaluate root growth.

Root growth models	df	AIC	Δ AIC
Initial size+Treatment+Species	5	27.07	0
Initial size+Treatment+Species+Species×Treatment	6	27.31	0.23
Initial size+Treatment+Species+Initial size×Treatment	6	28.04	0.96
Initial size+Treatment+Species+Species×Treatment+Initial size×Treatment	7	28.30	1.22
Initial size+Treatment+Species+Initial size×Species	6	29.02	1.94
Initial size+Treatment+Species+Species×Treatment+Initial size×Species	7	29.08	2.00
Initial size+Treatment+Species+Initial size×Species+Initial size×Treatment	7	29.89	2.81
Initial size+Treatment+Species+Initial size×Species+Initial size×Treatment	7	29.89	2.81
Initial size+Treatment+Species+Species×Treatment+Initial size×Species+Initial size×Treatment	8	30.21	3.13
Initial size+species	4	31.06	3.98
Initial size+treatment	4	40.68	13.60
Initial size	3	44.10	17.02
Treatment+species	4	129.32	102.25
Treatment	3	134.15	107.07
Species	3	162.28	135.20
Null	2	165.77	138.69

df=degrees of freedom, AIC=Akaike information criteria, Δ AIC=difference in AIC between each model and the best one.

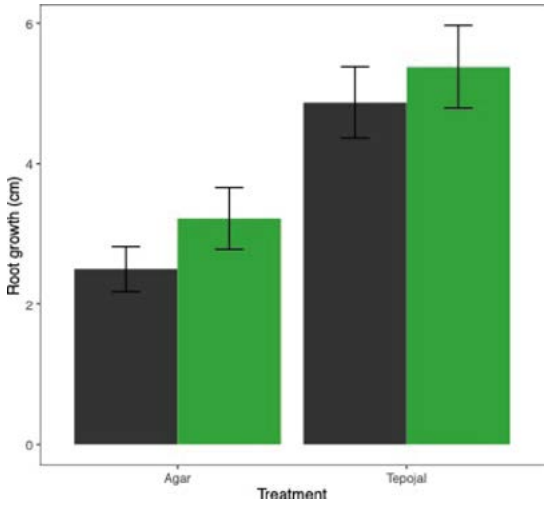

Figure 2. Root growth of *Barkeria uniflora* (green) y *B. scandens* (gray) cultivated *in vitro*.

all three independent variables that are present in the best model and differed only in the inclusion of the interaction of the variables.

Evaluation of the *ex-vitro* survival of *Barkeria uniflora* and *B. scandens*

Regardless of the species, survival at six months of culture *ex vitro* was higher in the seedlings that were cultivated in tepojal in *in vitro* conditions compared to those that were cultivated in agar. On average, the survival of the seedlings in tepojal was almost double compared to those from agar (Figure 3). The difference in survival of the treatment was greater in *Barkeria uniflora*, since it was almost three times greater for the seedlings that were previously cultivated *in vitro* in tepojal. According to the mixed effects linear models, using the jar as a random factor, we found that the best model is the one that only includes the treatment (Table 3).

Seed germination of *Barkeria whartoniana* in tepojal

Germination of *B. whartoniana* seeds were evaluated both in agar and in tepojal. In both culture media, *in vitro* seed germination occurred asynchronously, mainly between day 25 and day 28. At that time, green protocorms could already be recognized. The first leaves and roots emerged around day 42 after seed sowing. Seed germination observed in the

Table 3. Linear mixed effects models used to evaluate survival.

Survival Models	df	AIC	Δ AIC
Size	4	793.43	0
Treatment+Species	5	798.89	5.45
Treatment+Species+Treatment×Species	6	799.95	6.51
Null	3	840.74	47.30
Species	4	843.53	50.09

df=degrees of freedom, AIC=Akaike information criteria, Δ AIC=difference in AIC between each model and the best one.

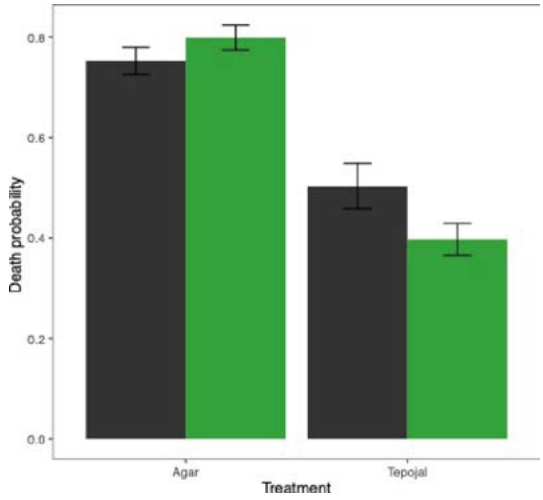

Figure 3. *Ex vitro* death probability of *Barkeria uniflora* (green) y *B. scandens* (gray) from plants cultivated *in vitro* with tepojal and agar.

liquid medium with tepojal was not apparently different from that in agar. In both cases the entire surface of the jar was covered with green protocorms.

DISCUSSION

This is the first study were the effect of tepojal *vs.* agar has been evaluated. At first, we considered that tepojal would not have better results than agar, since the latter is the most widely used gelling agent worldwide. But, considering the low cost and accessibility of tepojal, it would compensate for lower effectiveness compared to agar. Contrary to our expectations, tepojal produced better-than-expected results.

Effect of tepojal on growth of *Barkeria uniflora* and *B. scandens*

No differences were found in growth between agar and tepojal in both species (Figure 1 and 2). This result would be enough to consider tepojal as a good substitute for agar for aerial growth. But if we consider that cultivation in this volcanic rock uses a much less nutritious culture medium, it turns out that it is more efficient. Greater vegetative growth was obtained per gram of culture medium used; therefore, if we consider the cost of agar and the nutrient medium, the use of tepojal with liquid medium is much cheaper and much easier to obtain, especially for hobby *in vitro* growers.

Although it was found that the initial size of the plants and the species are decisive in the final length of the root, an important finding was that plants grown in tepojal had the most robust and longest roots. For *Barkeria* species, developing large roots is important because they do not have pseudobulbs and they can not store. It should be noted that even though the plants grown in tepojal had a liquid nutrient medium, the roots did not present hyperhydratability. Likewise, it was possible to observe that the seedlings grown in agar had less robust and shorter roots, their color was light green, somewhat transparent, somewhat slimy, and very fragile or brittle. In contrast, seedlings grown in tepojal, had roots with whitish in color, not very viscous and less

brittle. The whitish color of the root suggests that that they may already have a well-differentiated canopy.

Barkeria uniflora and *B. scandens* produced longer roots in the tepojal. Although, the models detected that the interactions between independent variables may be important in determining the length of the roots. This suggests that cultivation in tepojal can produce different effects between species and be influenced by the initial size of the plants. Thus, it is necessary to carry out more studies that involve other species of the genera, as well as plants of different sizes to evaluate the effect of cultivation in tepojal on root development.

Use of tepojal in the germination of *Barkeria whartoniana*

Originally, tepojal was not considered as a possible substitute for agar for the germination process of orchid seeds. A concern we had for the use of tepojal was that the coarse grain could cause the seeds to migrate to the bottom of the jars, causing seedlings to drown. For this reason, we initially used very fine tepojal sand, but the material turned out to be very compact. All plants growth in these jars occurred only at the surface of the culture medium, producing a cluster of roots and stems that could be detached very easily from the culture medium. Because of these issues, we performed the experiment again using slightly larger grains (2 to 3 mm in diameter). Even though the seeds could have gone to the bottom of the jar given the size of the tepojal particles, this did not occur very often because of the liquid culture medium that cause the seeds to float. Plants in this experiment produced roots that managed to penetrate the substrate. As a result, we obtained vigorous plants with well-developed roots, which developed for 3 to 4 months. Subsequently, a small additional experiment was carried out to see if it was possible to subcultivate seedlings that had been germinated in tepojal to a new jar with this same material but with a coarser grain, as well as to a culture medium with agar. We found that both subcultures are feasible, and that the seedlings develop normally. Most of the seeds that successfully germinated using this method are currently already growing *ex vitro* in the greenhouse.

An unexpected result of the use of tepojal sand for the germination of *Barkeria whartoniana* was found when we removed the plants for their subsequent subculture. It was observed that there were still some seeds that had not developed as protocorms. So, with a shaker, the surface of the sand was slightly stirred and rehydrated with liquid culture medium. After a few days, a second wave of protocorms, which grew and generate new seedlings, were observed. This is an additional advantage as more plants can be obtained with fewer steps. This contrasts with germination carried out in agar, where at the time of subculturing, the plants are removed with fragments of agar and in the end the flasks are left with almost no culture medium and are unusable for the development of the seeds that did not germinate.

Ex vitro* survival of *Barkeria uniflora* and *B. scandens

Survival after six months of *ex vitro* culture was almost twice as high in seedlings that were previously cultivated *in vitro* in tepojal than in agar (Figure 3). Apparently, this is because the seedlings cultivated in tepojal had longer and less brittle roots. Thus, they may be more viable from the moment they come out of the jar. In addition, roots from

the agar culture were more fragile. Also, it was much easier to extract from tepojal the seedlings with all their roots compared to agar. This was because agar adheres and wraps more firmly around the root, so it is necessary to remove it to avoid the growth of fungi and bacteria. In addition, although the roots may remain attached to the tepojal, it was not necessary to remove this substrate completely.

Survival was greater in *Barkeria uniflora* than *B. scandens* on tepojal. This result implies that species can respond differently to subcultivation after growing in tepojal. This finding was the result of using two species at the same time for the experiment, a situation that is rare in *in vitro* culture experiments. It is not clear why this difference was found between the two species. There may be some explanations of the higher survival in initial stages of *B. uniflora* compared to *B. scandens*. One of them is that *in vitro* development is more vigorous in *B. uniflora*, and it has previously been observed that larger plants are more successful in surviving, even under natural conditions (Segovia-Rivas *et al.*, 2018). The faster growth of *B. uniflora* could be associated with its life history strategy (Halbinger 1977), since it behaves like a species capable of colonizing slightly disturbed environments (ruderal), such as guava crops, while *B. scandens* can be considered a stress tolerant plant. These differences in life histories were reflected by several *B. uniflora* plants flowering during *in vitro* culture, while none of the *B. scandens* plants did. Therefore, it is important to understand the biology associated with the species used, since this can affect the results obtained in the acclimatization of the plants.

Because with the use of tepojal a little water stress can be applied before removing the plants from the jar, it is possible that the orchid seedlings are more "hardened" and therefore may have greater ex vitro survival compared to plants grown on agar. The development of this process can substantially increase the survival of the plants in one of the most critical steps in the production of orchids where mortality is higher (Suárez-Quijada *et al.*, 2007). This experiment helped to obtain experiences in subcultivation and techniques have subsequently been developed that have allowed for greater survival of Barkeria seedlings and other groups of orchids.

FINAL CONSIDERATIONS

The use of tepojal is novel because it does not involve the gelation phase, and this apparently generated some additional advantages in *ex vitro* survival. This technic could increase *in vitro* culture of orchids, since it is easier to obtain for hobbyists who do not have agar suppliers, especially in small quantities and in remote places. In addition, tepojal reduces production costs because less nutrient solution is used per volume of culture medium and has similar or even better results than agar (Romay *et al.*, 2006). Another advantage is that time in laboratory facilities is reduced because it is not necessary to wait for the media to gel and resuspension. When using tepojal, it is enough to wait for the medium to cool before it can be used. It is also possible to reduce costs in subcultures because it is enough to hydrate the medium with a nutrient solution when it is cultivated in tepojal without having to repeat the entire sowing procedure. Additionally, during *in vitro* culture, it is possible to redistribute the nutrients and conditioners in the culture medium by gently decanting the flasks, particularly for the less soluble ones such as activated carbon.

This study is a pioneer in the use of tepojal as a substitute for agar. It has given us very encouraging results for its widespread use but there is still much to be investigated. Particularly, it will be necessary to evaluate how other species and other genera react to *in vitro* culture in tepojal. It is also necessary to evaluate the entire rehydration process with nutrient solutions because the adequate amounts of nutrients and conditioners of the culture medium that must be incorporated are unknown. An aspect that needs to be resolved is the process of cleaning and sterilization of tepojal to reduce the use of water and energy. In this study, the tepojal was autoclaved two times in total to avoid contamination. The use of energy and time in this process is still high and other alternatives will have to be found in the future. However, despite all these possible drawbacks, we may conclude that the use of tepojal as a substrate in *in vitro* culture is highly recommended.

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