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Micropropagation of orchids in plastic containers with non-autoclavable media

Micropropagación de orquídeas en recipientes plásticos con medios sin autoclave

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ABSTRACT

The ornamental importance of orchids is due to the various shapes, sizes, and colors of their flowers. Unfortunately, the asexual and sexual propagation of these species takes a long time and produces a limited number of commercial propagules. Thus, plant tissue culture has been a suitable alternative for micropropagation. However, some of the materials used in this technique are expensive. Therefore, this study aimed to propose an alternative method for in vitro propagation that involves the use of disposable containers and a culture medium that requires no autoclaving. Different concentrations of 6-benzylaminopurine (BAP: 0, 0.5, 1.0, 1.5, 2.0, and 2.5 mg•L-[1]) in MS (Murashige and Skoog) medium autoclaved and non-autoclaved were assessed, followed by acclimatization. After 45 days of incubation, assessed the percentage of contamination, number of shoots per explant, shoot length, number of leaves, number of roots, and root length. No contamination using the alternative method and autoclave was observed. The highest number of shoots per explant (3.77) was produced in 1.0 mg•L-[1] BAP in non-autoclaved culture medium. A 98 % survival rate was observed during the acclimatization phase. These results can be used as an alternative for the commercial micropropagation of orchids.

KEY WORDS: Non-sterile medium, plastic packaging, commercial propagation.

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RESUMEN

La importancia ornamental de las orquídeas se debe a las diversas formas, tamaños y colores de sus flores. Desafortunadamente, la propagación asexual y sexual de estas especies lleva mucho tiempo y produce un número limitado de propágulos comerciales. Por tanto, el cultivo de tejidos vegetales ha resultado una alternativa adecuada para la micropropagación. Sin embargo, algunos de los materiales utilizados en esta técnica son caros. Por lo tanto, el objetivo de este estudio fue proponer un método alternativo para la propagación in vitro que involucra el uso de recipientes desechables y un medio de cultivo que no requiere autoclave. Se evaluaron diferentes concentraciones de 6-bencilaminopurina (BAP: 0, 0.5, 1.0, 1.5, 2,0 y 2.5 mg•L-^{1}) en medio MS (Murashige y Skoog) autoclavado y no autoclavado, seguido de aclimatación. Luego de 45 días de incubación, se evaluó el porcentaje de contaminación, número de brotes por explante, longitud de brotes, número de hojas, número de raíces y longitud de raíces. No se observó contaminación utilizando el método alternativo y autoclave. El mayor número de brotes por explante (3.77) se produjo en 1,0 mg•L-^{1} de BAP en medio de cultivo no autoclavado. Se observó una tasa de supervivencia del 98 % durante la fase de aclimatación. Estos resultados pueden utilizarse como una alternativa para la micropropagación comercial de orquídeas.

PALABRAS CLAVE: Medio no estéril, envases plásticos, propagación comercial.

Introduction

Micropropagation is a technique that allows producing a large number of plants over a short time and in small facilities (George, 2008; Pastelin-Solano *et al.,* 2020). This technique has been successfully used in the *in vitro* propagation of several species of orchids (Menchaca-García *et al*., 2012; Yam & Arditi, 2017). However, these techniques involve expensive sophisticated equipment, which increases production costs. Some examples are the autoclave use for culture medium sterilization, together with specialized containers, such as test tubes, glass flasks, bioreactors, and others (Salgado & Peñaranda, 2019; Martínez-Rivero *et al.,* 2020). For this reason, alternatives for the micropropagation of orchids are being explored to reduce costs, as well as to facilitate a part of the micropropagation process through the use of plastic packaging.

The sterilization of culture media can be performed either by autoclaving with specialized equipment or chemically (Martínez-Rivero *et al*., 2020). However, autoclaves are relatively expensive and their use involves high consumption of electricity because the operating principle is based on heating a resistor through electrical power to achieve the proper temperature and

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pressure (Ormeño-Orrillo & Dávila, 1999; DeSantis, 2021). It is mentioned that during the orchids micropropagation , the expenditure on electrical energy amounts to 23 % of the total production cost (Chen, 2016). It is mentioned that the electrical energy consumption of the autoclave amounts to 3 % of the production cost. On the other hand, the use of glass containers is limited by their availability and market price, making these unsuitable for commercial micropropagation, hence the need to implement the use of containers made of other materials (Sáez *et al*., 2012; Vahdati *et al*., 2017). For this reason, alternatives are being assessed to overcome these constraints, such as the use of low-cost, easily accessible chemical sterilants for the culture medium, instruments, and culture vessels (Quiala *et al*., 2002; Martínez-Rivero *et al*., 2020). Sodium hypochlorite (NaClO) is frequently used as a disinfectant and can be used to sterilize culture vessels and media used for micropropagation (Pais *et al*., 2016; Suaib *et al*., 2018).

The genus *Cattleya* has 114 economically important species for their large flowers and multiple colors (van den Berg, 2014; Caballero-Villalobos *et al*., 2017). In this regard, research focused on the development of commercial hybrids with appealing ornamental characteristics has been conducted (Cardoso *et al*., 2016; van den Berg, 2019). A hybrid with attractive features is *Rhyncholaeliocattleya*. Like most orchids, it has propagation limitations due to the long time it takes to produce pseudobulbs (Murguía *et al*., 2016; Murthy *et al*., 2018). While sexual reproduction (through seeds) is limited by its naturally low germination rates (Shao *et al*., 2017; Yeh *et al*., 2021). Therefore, this study aimed to propose an alternative method for the *in-vitro* propagation of *Rhyncholaeliocattleya*, involving the use of disposable bottles and a culture medium that requires no autoclaving.

Material and Methods

Plant material and asymbiotic germination

One capsule of approximately 8 months of maturity of the commercial hybrid *Rhyncholaeliocattleya* (*Rlc*) was collected. The capsule was obtained through assisted self-pollination; it was pretreated by several washing steps with a commercial detergent and rinsed with running water. Subsequently, the capsule was transferred to the laminar flow cabinet, where it was immersed in 96° ethanol for 1 minute under constant stirring, then removed with forceps for flaming; this step was repeated three times. Afterward, longitudinal cuts were made in the capsule with a scalpel to extract the seeds. These were sowed in MS medium (Murashige & Skoog 1962) supplemented with 30 g L^{-1} sucrose and 2.5 g•L^{-1} Phytagel® as a gelling agent. A volume of 20 mL was transferred to each jar and autoclaved at 1.5 kg•cm^{-2} and 121°C for 20 min. Finally, culture jars were incubated at 25 ± 2°C under irradiation of 50 µmol•m^{-2}•s^{-1} provided by LED lamps.

Propagation

The material used in this work consisted of *Rlc* seedlings germinated *in vitro* (individual shoots measuring ~ 1 ± 0.2 cm in length). These seedlings were transferred to MS medium supplemented with 30 g•L^{-1} sucrose and 2.5 g•L^{-1} Phytagel® as a gelling agent. Different concentrations of

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6-benzylaminopurine (BAP: 0, 0.5, 1.0, 1.5, 2.0, and 2.5 mg•L^{-1}) as a plant growth regulator (PGR) were tested. After 4s days of culture, the variables evaluated were contamination percentage, shoot number, shoot length, number of leaves per shoot, root number, and root length.

Autoclave method

A volume of 20 mL was transferred to each glass jar and autoclaved at 1.5 kg•$cm^{-2}$and 121°C for 20 min (Figure 1A). The jars were covered with aluminum foil and sealed with a self-adhering film. Finally, culture jars were incubated at 25 ± 2°C under irradiation of 50 µmol•m^{-2}• s^{-1} provided by LED lamps.

Non-autoclave method

20 mL of medium were poured into each disposable plastic polypropylene (PP) container (Figure 1B-C). The culture medium and containers were not autoclaved; instead, the following procedure was used for sterilization. The plastic containers (250 mL capacity thermoplastic polyolefin containers (food grade plastic)) and the respective lids were washed with soap and water and left to dry. In the laminar flow hood, the jars and lids were rinsed on a plastic tray with a 3 % NaClO (v/v) solution (household bleach with 6 % active ingredient). Afterward, a clean flannel was moistened with this NaClO solution and placed on top of the jars and lids upside down, they were left in the laminar flow hood until they dried (this type of sterilization is carried out through NaClO gases). They were then covered and kept under sterile conditions. The culture medium was sterilized by heating to the boiling point (100 °C) for 1 min. Then, inside the laminar flow cabinet, 20 mL of culture medium was transferred to each disposable container, and the plant material was planted. The containers were sealed with with their lids a self-adhering film. Finally, jars were incubated at 25 ± 2°C under irradiation of 50 µmol•m^{-2}•s^{-1} provided by LED lamps.

Rooting and Acclimatization

Orchid shoots produced roots during the propagation phase (experiment with BAP); therefore, a rooting phase was unnecessary. 120 *Rlc* plants were used for acclimatization. Shoots measuring 3 cm in length and with adequate root development were rinsed with running water to remove any residues from the culture medium. Later, they were sown in a substrate composed of volcanic rock (tezontle) and agrolite (type of perlite) (1:1 v/v) (Leyva-Ovalle *et al*., 2020), which had been previously sterilized by sun exposure for 72 h; 60-well plastic trays with lids were used. After shoots were sown, the plastic lid was placed atop each tray to maintain a high relative humidity; shoots were sprayed with water once a week for 14 days. After this period, the plastic lid was removed and plantlets were watered with running water twice a week and fertilized with 1 g•L^{-1} Go Green® once a week. The percentage of survival was evaluated after 60 days of culture.

Statistical Analysis

A completely randomized experimental design was applied for all experiments, including three replicates. 20 shoots per treatment were used in each of the replicates. The data obtained

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were statistically processed with IBM SPSS Statistics software (version 21). An ANOVA followed by a Tukey's test ($p \leq 0.05$) was performed to determine whether the differences between treatments were statistically significant.

Results and Discussion

Asymbiotic germination

After 45 days of culture, germination was observed in approximately 100 % of the seeds planted.

Autoclave method vs non-autoclave method

For propagation (using individual shoots), no contamination was observed in the disposable containers using the autoclave method and non-autoclave method. In general, the variables evaluated were higher in the non-autoclave method than in the autoclave method (Figure 1) (Table 1).

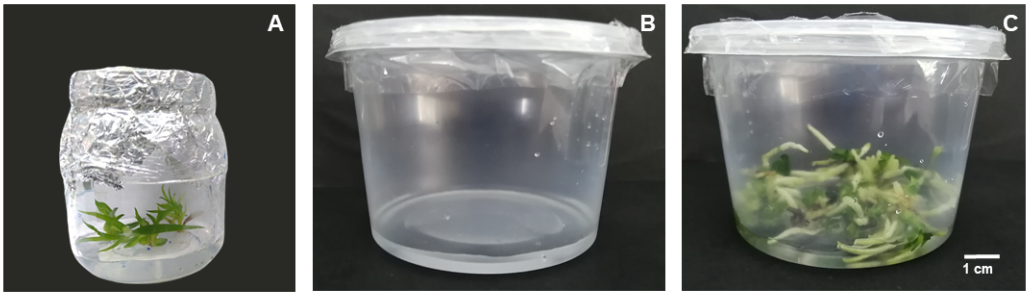

Figure 1. *In vitro* propagation of *Rhyncholaeliocattleya*. A) Glass jars with autoclaved culture medium, B) Plastic containers with non-autoclaved culture medium, B) Plastic containers used in micropropagation of Rlc. Source: Own elaboration based on results.

The highest number of shoots per explant (3.77) was obtained in non-autoclaved culture medium supplemented with 1.0 mg•L^{-1} BAP (Figure 2E), followed by 3.50 in non-autoclaved culture medium supplemented with 0.5 mg•L^{-1} BAP. 3.14 shoots in non-autoclaved culture medium supplemented with 1.5 mg•L^{-1} BAP (Figure 2F). The lowest number of shoots per explant (1.52) was observed in the autoclaved culture medium without BAP (Figure 2A).

On the other hand, shoot length was 3.26 cm in non-autoclaved culture medium supplemented with 1.5 mg•L^{-1} BAP (Figure 2F), followed by 2.47 cm in non-autoclaved culture medium cm with 1.0 mg•L^{-1} BAP (Figure 2E). The lowest shoot length (1.23 cm) was observed in

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the autoclaved culture medium without BAP (Figure 2A). The highest number of leaves was 2.56 cm in non-autoclaved culture medium supplemented with 1.5 mg•L^{-1} BAP (Figure 2F), followed by 2.23 cm in autoclaved culture medium cm with 1.5 mg•L^{-1} BAP (Figure 2C). The lowest shoot length (1.22) was observed in the autoclaved culture medium supplemented with 2.5 mg•L^{-1} BAP. For roots, the treatment supplemented with 1.5 mg•L^{-1} BAP showed the highest results: 2.57 roots per explant measuring 2.39 cm in length (Figure 2E). In contrast, autoclaved culture medium cm with 2.5 mg•L^{-1} BAP showed the lowest results (Table 1).

Table 1. Effect of different concentrations of BAP and sterilization method on in vitro multiplication of RIc.

BAP concentration (mg•L^{-1})	Number of shoots	Shoot length (cm)	Number of leaves	Number of roots	Root length (cm)
			Autoclave method		
0	1.52 ± 0.26^{d}	1.23 ± 0.12^{c}	1.30 ± 0.09^{cd}	1.44 ± 0.21^{c}	1.48 ± 0.29^{b}
0.5	2.46 ± 0.21^{c}	1.25 ± 0.10^{c}	1.35 ± 0.11^{cd}	2.00 ± 0.22^{bc}	1.68 ± 0.21^{b}
1.0	2.58 ± 0.24^{c}	1.86 ± 0.14^{b}	1.85 ± 0.10^{bc}	2.10 ± 0.17^{bc}	2.16 ± 0.26^{a}
1.5	2.09 ± 0.12^{d}	2.15 ± 0.19^{b}	2.23 ± 0.11^{b}	2.63 ± 0.18^{b}	2.28 ± 0.15^{a}
2.0	2.17 ± 0.19^{c}	2.05 ± 0.15^{c}	1.68 ± 0.08^{c}	1.98 ± 0.24^{c}	1.14 ± 0.17^{c}
2.5	1.82 ± 0.14 ^{d}	1.28 ± 0.07^{c}	1.22 ± 0.13^{d}	1.05 ± 0.19^{c}	1.09 ± 0.20^{c}
			Método sin autoclave		
0	2.57 ± 0.20^{c}	1.65 ± 0.13^{c}	1.41 ± 0.10^{c}	1.60 ± 0.24^{c}	2.00 ± 0.32^{a}
0.5	3.50 ± 0.22^{b}	1.64 ± 0.11^{c}	1.59 ± 0.09^{c}	2.14 ± 0.25^{bc}	1.73 ± 0.21^{a}
1.0	3.77 ± 0.27^{a}	2.47 ± 0.12^{b}	1.92 ± 0.12^{b}	2.50 ± 0.22^{b}	2.36 ± 0.26^{a}
1.5	3.14 ± 0.14^{c}	3.26 ± 0.22^{a}	2.56 ± 0.13^{a}	3.57 ± 0.20^{a}	2.39 ± 0.27^{a}
2.0	2.88 ± 0.26^{c}	2.34 ± 0.17^{b}	1.84 ± 0.10^{bc}	3.00 ± 0.30^{ab}	1.41 ± 0.14^{b}
2.5	2.50 ± 0.18^{c}	1.44 ± 0.11^{c}	1.52 ± 0.10^{c}	1.25 ± 0.25^{c}	1.20 ± 0.20^{b}

The mean ± standard error is represented. Different letters express significant differences (Tukey, $p \leq 0.05$).
Source: Own elaboration based on results.

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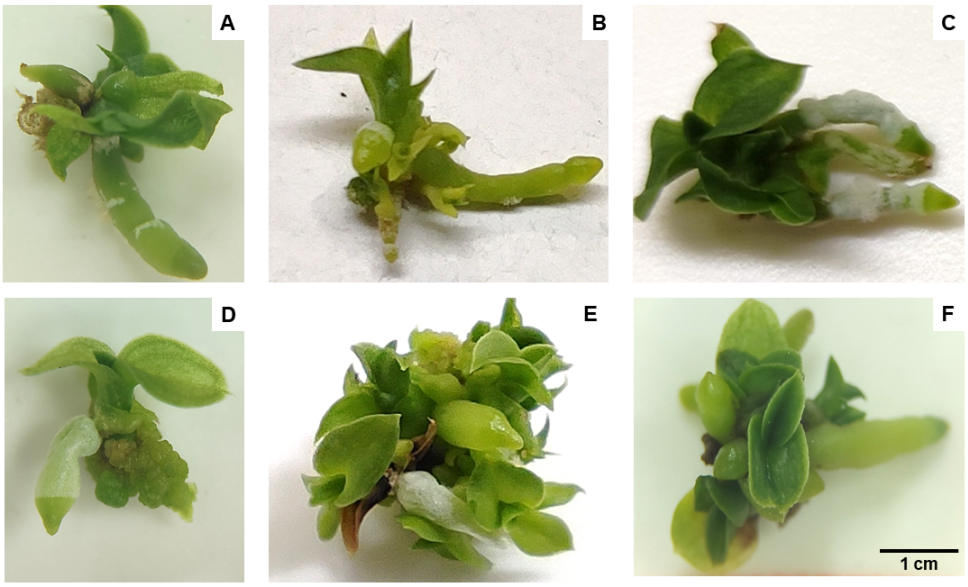

Figure 2. Effect of 6-benzylaminopurine and sterilization method on the multiplication of Rhyncholaeliocattleya. A-C) Autoclaved culture medium (0, 1.0 and 1.5 mg•L^{-1} BAP, respectively), D-F) Non-autoclaved culture medium (0, 1.0 and 1.5 mg•L^{-1} BAP, respectively). Source: Own elaboration based on results.

In orchids, micropropagation has been an alternative to produce many commercial propagules (Bhattacharyya *et al*., 2018; Leyva-Ovalle *et al*., 2020). In this sense, the production of a high number of orchid plants of ornamental interest will bring a greater economic profit for producers (Murthy *et al*., 2018). The proliferation of different orchid species has been achieved mainly through supplementation with 6-Benzylaminopurine (BAP). Pastelin-Solano *et al*. (2020) micropropagated *Vanilla planifolia* with 2 mg•L^{-1} BAP, producing 7.9 shoots per explant. Souza *et al*. (2021) micropropagated *Cattleya crispata* with 0.5 mg•L^{-1} BAP, obtaining 5 shoots per explant. These findings are consistent with our results because we obtained 3.77 shoots per explant of the *Rhyncholaeliocattleya* using medium supplemented with 1 mg•L^{-1} BAP. This indicates that BAP is a PGR that can be used in the micropropagation of different species of orchids, particularly this hybrid of the genus *Cattleya*.

The non-autoclaved culture medium had higher values in the variables evaluated compared to the autoclaved culture medium. Martínez-Rivero *et al*. (2020) mentioned that in the culture medium that did not undergo autoclaving conditions (high temperatures and high pressure), there was less degradation of nutrients and growth regulators. Harabi *et al*. (2016) designed a ceramic filter useful in the sterilization of the culture medium used in the micropropagation of plants, concluding that not heating the culture media during its sterilization contributes to preserving the quality of thermolabile organic substances.

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Various types of containers can be used for the *in vitro* propagation of orchids (glass bottles, magenta boxes, test tubes, etc.) (Sáez *et al*., 2012; Vahdati *et al*., 2017; Singh, 2018; Gago *et al*., 2021). However, the availability of these containers limits the *in-vitro* propagation of these cultures. Therefore, the present study shows the suitability of disposable plastic bottles for this purpose; this could translate into lower production costs. In our study, the use of sodium hypochlorite allowed the correct disinfection of the culture vessels. Weber *et al*. (2015) determined that 0.1 % v/v NaClO worked to sterilize containers by immersing lids and bottoms, for potato micropropagation.

The material of the containers used for *in vitro* cultures is of the utmost importance due to the light diffusion through them, which should allow cultured shoots to display a better morphogenetic response (Emara *et al*., 2018; Sarikhani *et al*., 2021). In this sense, the present study showed that disposable plastic containers allowed an adequate diffusion of light radiation, which produced a normal morphogenetic response.

Furthermore, the operating costs of a plant-tissue culture laboratory, including electricity, increase production costs (Cardoso *et al*., 2018; Dhiman *et al*., 2021). To reduce energy consumption, this study evaluated a culture medium that was not autoclaved (electrically) but rather sterilized by boiling. As well as bottles sterilized by chemical sterilization (NaClO vapor). This prevented the contamination of the culture media and made possible the micropropagation of the *Rhyncholaeliocattleya*. This finding is extremely relevant, as the sterilization method assessed represents savings in energy expenditure during *in-vitro* propagation processes.

Rooting and Acclimatization

During the multiplication phase, orchids produced roots (Figure 3A); therefore, a rooting stage was unnecessary. With regard to acclimatization, 98 % survival was observed, regardless of whether they came from the autoclaved or non-autoclaved medium (Figure 3B).

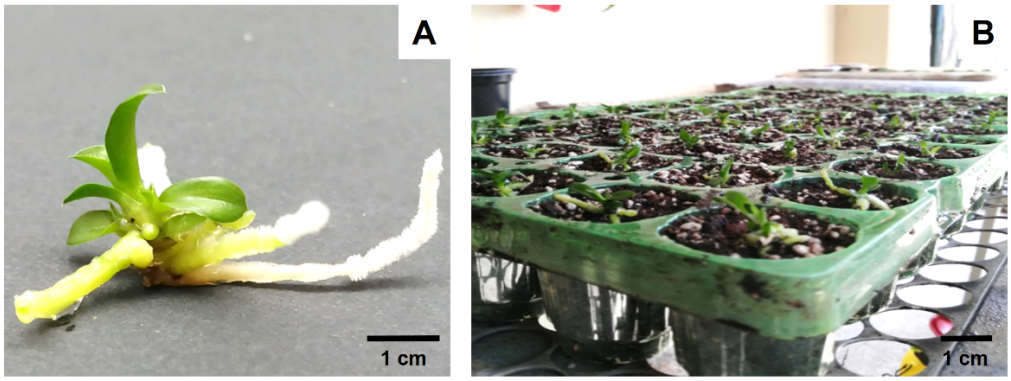

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Figure 3. Acclimation of Rlc. A) In vitro plants in the multiplication phase; B) Plants in the acclimatization process 30 days.

Source: Own elaboration based on results.

As reported by Manokari *et al*. (2021), plants need root systems for proper nutrient absorption, which involves facilitation to ensure plant survival. The roots can become attached to a structure that provides physical support, such as bark, wood, or bamboo stick; besides, in orchids, photosynthesis also takes place in roots, so the presence of these structures is hugely important (Ma *et al*., 2019). Acclimatization is considered the most critical phase in the micropropagation process because it is the last step that defines the success of the entire process (Hazarika, 2003; Naaz *et al*., 2019). A large number of authors mention that acclimatization is one of the most complex stages and that low survival rates are generated (Varshney & Anis, 2012; Indacochea-Ganchozo *et al*., 2017; Valencia-Juárez *et al*., 2019). In the present study, we achieved 98 % survival of plants produced through tissue culture. Although successful cases of acclimatization have been reported in a wide variety of orchids, it is important to note that each of these species requires a particular protocol to ensure the survival of the plants obtained. Nowadays, cases of micropropagation of orchids of the genus *Cattleya* have been reported, such as the study by Menezes-Sá *et al*. (2021), who propagated *Cattleya tigrina in vitro*, and Mantovani *et al*. (2017), who micropropagated *Cattleya guttata* using silicon and sodium silicate**.**

Conclusions

This study proposes alternative techniques for the micropropagation of orchids (*Rhyncholaeliocattleya*) that could be used in the large-scale production and propagation of various types of hybrids of this genus. The results of the present work support the feasibility hypothesis of establishing these alternative techniques for *in vitro* micropropagation, i.e., the use of 1 mg•L^{-1} of BAP, plastic culture containers sterilized with a sodium hypochlorite solution, and the sterilization of the culture medium with no autoclaving (simply by boiling).

Authors' contribution

Ramírez-Mosqueda: Work conceptualization, Research, Methodology, Writing the Original Draft, and Writing-Revision and Editing.

All authors of this manuscript have read and accepted the published version of the manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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