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Article

A protocol for the *in vitro* establishment of *Psidium guajava* L. using seeds, for the conservation of native germplasm

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Abstract

The guava *Psidium guajava* L. is a very important fruit tree for Aguascalientes. In the municipality of Calvillo, guava orchards were established with an area of more than 6 000 ha. The current problem is that it is being replaced with other crops, such as avocados, lemons, and vegetables. One solution for the conservation of these important genetic resources is germplasm banks, linked to *in vitro* propagation, which is a biotechnological tool that allows healthy guava plants to be reproduced in small spaces and controlled conditions. In view of this, a methodology for the *in vitro* establishment of guava seeds was standardized. Four treatments were evaluated in a first experiment to improve aseptic conditions, then a second experiment was conducted to determine the best *in vitro* culture proposal, including scarified and non-scarified seeds to define the complete protocol. In the first experiment, T3 with 0.1% Tween® 20 (polysorbate), 6 ml L^{-1} Fractal® (citrus seed extract), 5% chlorine, and 70% ethanol showed 95% asepsis. In the second experiment, from the *in vitro* establishment stage, T4, with seed scarified with activated charcoal, obtained 90% germination with a length of 4.05 cm. Finally, it was found that, using scarified seed, the MS culture medium supplemented with 2 g L^{-1} of activated charcoal, 0.5 mg L^{-1} of BAP and 7.5 g L^{-1} of agar-agar was the best to establish the *in vitro* reproduction protocol.

Keywords:

asepsis, conservation, guava, *in vitro* culture.

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Introduction

In Mexico, there is an established guava area of 20 044 ha, with a production of 406 000 t year^{-1}, which generates an economic spillover of USD $144.41 million (Ramos-Sandoval *et al*., 2017). Aguascalientes ranks second in production in Mexico, with an established area of 5 950 ha and an annual production of 126 392 t with a commercial value of USD $39.31 million (SIAP, 2025). The type of soil in Calvillo, Aguascalientes, contributes to the production of sweet guavas, and this advantage means that consumers preferentially demand the fruit throughout the country (Ramirez *et al*., 2022).

In another sense, the *in vitro* establishment of crops using seeds offers advantages, such as providing seedlings that serve as a source of explants to carry out micropropagation and the conservation of native genetic variability, among others. In the establishment, the seeds use physical or chemical methods of scarification.

Physical methods are recommended to avoid using toxic substances that pollute the environment. *In vitro* propagation is reported as successful in trees of forest importance, increases the germination rate and reduces the time in which it occurs (Zurita-Valencia *et al*., 2014), and is carried out by selecting an explant (apical meristem) that is planted in a nutritious culture medium to achieve a morphogenic response and form a complete plant from a tissue or organ (cell totipotency).

The ability of plant cells to regenerate plants is also used for germplasm conservation. Likewise, *in vitro* morphogenesis is divided into embryogenesis and organogenesis (Torres *et al*., 2023). *In vitro* tissue culture systems are very important in plant biotechnology. Regarding *in vitro* establishment, seeds are used to facilitate the asepsis stage and establish tissues free of fungi, viruses, and bacteria. Light intensity is also vital for tissue development (Ruíz-Rivas *et al*., 2022).

Guava has been propagated *in vitro* by organogenesis and somatic embryogenesis, the main problem being phenolic oxidation in the establishment and contamination in the multiplication stage; overcoming these barriers, tissues can be established for mass propagation (Domínguez-Perales *et al*., 2016). On the other hand, *in vitro* germplasm banks are useful because they are used for the conservation of plant genetic resources of interest through the propagation of tissues and seeds of plant species.

The purpose is to have and conserve the greatest genetic diversity and over time regenerate the desired genotypes. The collection unit is kept under controlled conditions in small spaces free of contamination by pathogenic microorganisms (Sánchez-Chiang and Jiménez, 2010).

In addition to the above, slow-growing culture is low-cost and allows the preservation of shoots *in vitro* because it decreases their metabolism and reduces the growth rates of vegetable crops; *in vitro* germplasm banks also serve to recover threatened or endangered plants; not all species can be conserved by seed; therefore, plant tissue culture is a very important option to maintain this gene pool for long periods for its conservation (Martínez, 2021).

Micropropagation is also used to carry out physiological studies of rejuvenation and aging to obtain productive plants or for conservation (Pérez *et al*., 2002). Plant biotechnology through plant tissue culture and *in vitro* germplasm banks contribute to the conservation of plant species of great importance, such as guava (Valdés-Infante *et al*., 2012). For the above, the objective was to standardize a methodology for *in vitro* establishment from guava seeds.

Materials and methods

Experiment location

The experiment was carried out at the Applied Biotechnology Laboratory of the El Llano Aguascalientes Technological Institute (ITEL, for its acronym in Spanish) in El Llano, Aguascalientes, Mexico, located at km 18 of the Aguascalientes-San Luis Potosí highway, at 21° 49’ 8.21” north latitude, 102° 10’ 02.96” west longitude at 2 008 masl (Google Earth, 2024).

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Plant material

Guava seeds of the 'Media China' type were obtained from 20 ripe fruits harvested in a commercial orchard located in San Tadeo, Calvillo, Aguascalientes at 21° 55' 13" north latitude, 102° 42' 09" west longitude at 1 699 masl (Google Earth, 2024). The handling of the plant material was carried out with tweezers and scalpels previously disinfected by immersion in 100% ethanol and flaming with a burner.

Characterization of the research work

The methodological structure was divided into two experiments with three stages.

First experiment

Asepsis and contamination control

The design used was completely randomized with four treatments and 10 replications, and the experimental unit was a 250 ml bottle with 10 seeds with MS medium (Murashige and Skoog, 1962). The treatments were: T1) Tween® 20 (polysorbate) at 0.1% + 5% chlorine + 50% ethanol; T2) Microdyn® (ionized silver) + 5% chlorine + 70% ethanol; T3) Tween® 20 at 0.1% + Fractal® (citrus seed extract) 6 ml L^{-1} + 5% chlorine and 70% ethanol, and T4) control without application.

Data were recorded 30 days after establishing the experiment. The study variable was the percentage of asepsis (%), for which the number of uncontaminated bottles was considered. The bottles that presented fungi, such as *Aspergillus* sp. and *Alternaria* sp., and bacteria, such as *Pseudomonas* sp., were considered as contaminated. For data analysis, the statistical package Statistica Version 13.3 (TIBCO Inc, 2017) was used for the analysis of variance (Anova) and Tukey's test at 5% for the comparison of means.

Physical scarification of seeds

Scarification is a physical or chemical process that breaks, scratches, mechanically alters or softens the seed coats to make them permeable and facilitate their germination. This part was performed with a physical procedure immediately after the asepsis stage. A sterile nail clipper was used to remove the testa of each seed in a laminar flow hood to prevent contamination. Sterile distilled water was used to make rinses and sterile paper towels were used to dry the seeds.

Second experiment

In vitro establishment

The design used was completely randomized with four treatments and 10 replications, and the experimental unit was a bottle with 6 guava seeds with MS medium. The treatments were: T1) control - non-scarified seed; T2) scarified seed; T3) non-scarified seed with activated charcoal 2 g L^{-1} and T4) scarified seed with activated charcoal 2 g L^{-1}. The MS culture medium was supplemented with 0.5 mg L^{-1} Benzyl aminopurine (BAP), 30 g L^{-1} sucrose and 7.5 g L^{-1} agar-agar. The conditions of the incubation room were: temperature 25 ±2 °C, a photoperiod of 16 h light and 8 h darkness and a light intensity of 2 000 lux.

The variables evaluated were: 1) germination percentage (%) and 2) shoot length (cm), the measurement was made 45 days after planting, a germinated seed was considered to be one with the radicle emerging to a length of 2 mm. To analyze the data from this experiment, the Anova test and Tukey's comparison of means test ($p \leq 0.05$) were performed using the statistical package Statistica Version 13.3 (TIBCO Inc, 2017).

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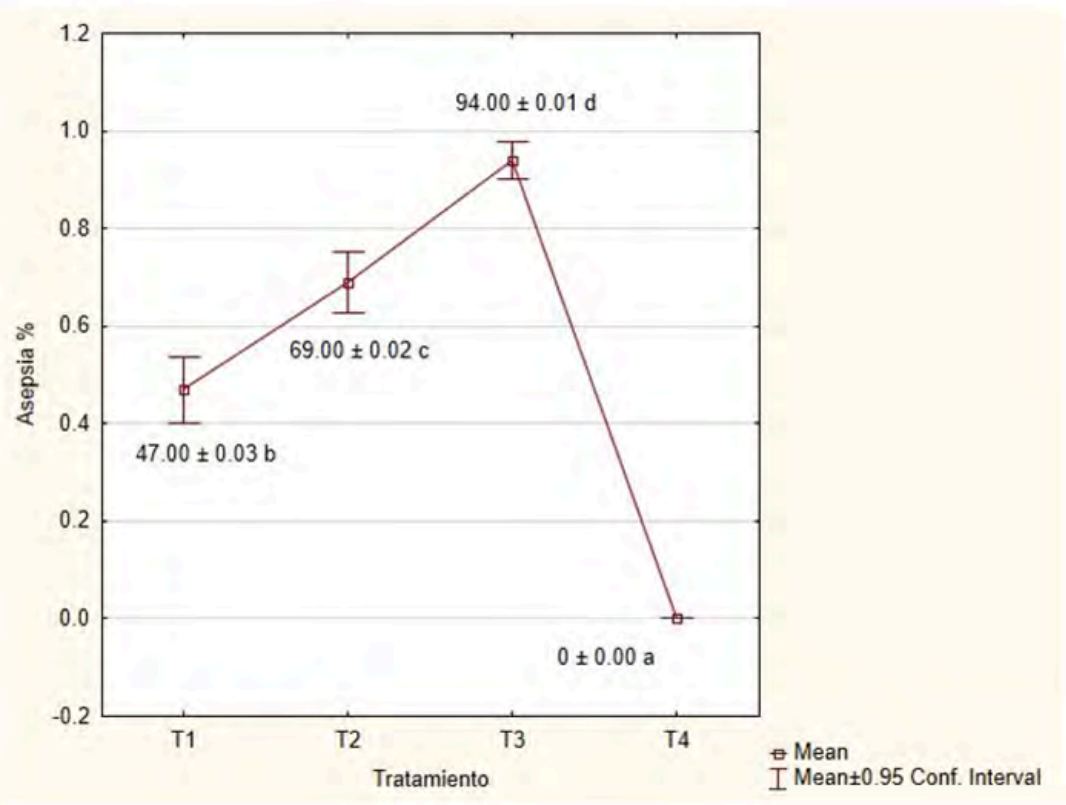
Results and discussion

First experiment

Asepsis and contamination control

In this variable, there were statistically significant differences, Anova ($p \leq 0.05$), in the treatments evaluated for the disinfection of guava seeds. The best results (Tukey $p \leq 0.05$) were in treatment T3 (without contamination by pathogens), whereas in T4, all the bottles were contaminated (Figure 1), which corroborates that the asepsis part is essential to establish *in vitro* cultures for mass regeneration under controlled conditions, such as photoperiod and temperature.

Figure 1. Results of Tukey's comparison of means ($p \leq 0.05$) of the first experiment (asepsis). Means with the same letter are statistically equal, n= 10 (Tukey, $p \leq 0.05$); HSD= 0.024; T1) Tween 20 (0.1%) + chlorine 5% + ethanol 50%; T2) Microdyn+ chlorine 5% + ethanol 70%; T3) Tween 20 (0.1%) + Fractal® 6 mL L^{-1} + chlorine 5% + ethanol 70%; T4) control without application.



In this regard, several previous studies on *in vitro* propagation of guava conclude that the critical stage to overcome is to obtain aseptic material for its establishment in the laboratory (Ocampo and Núñez, 2007). Continuing with T3, Fractal® (citrus extract, water-soluble liquid) acted as a bactericide and fungicide, functioning as a broad-spectrum organic disinfectant of germicidal action in the experiment. It is characterized by being biodegradable, with a development to resistance record, and is recommended in the prevention and treatment of foliar diseases in various cultivated plants (Berni Labs, 2025).

The concentration of chlorine (5% sodium hypochlorite) for 15 min to control the contamination of the explants is also very effective, as reported by the results of this research, coinciding with what has been reported by various authors (Ramírez and Salazar, 1997; Casanova *et al*., 2019). Other authors use more aggressive substances for the disinfection process, such as mercury bichloride, which is toxic to humans and the environment (Concepción et al., 2004).

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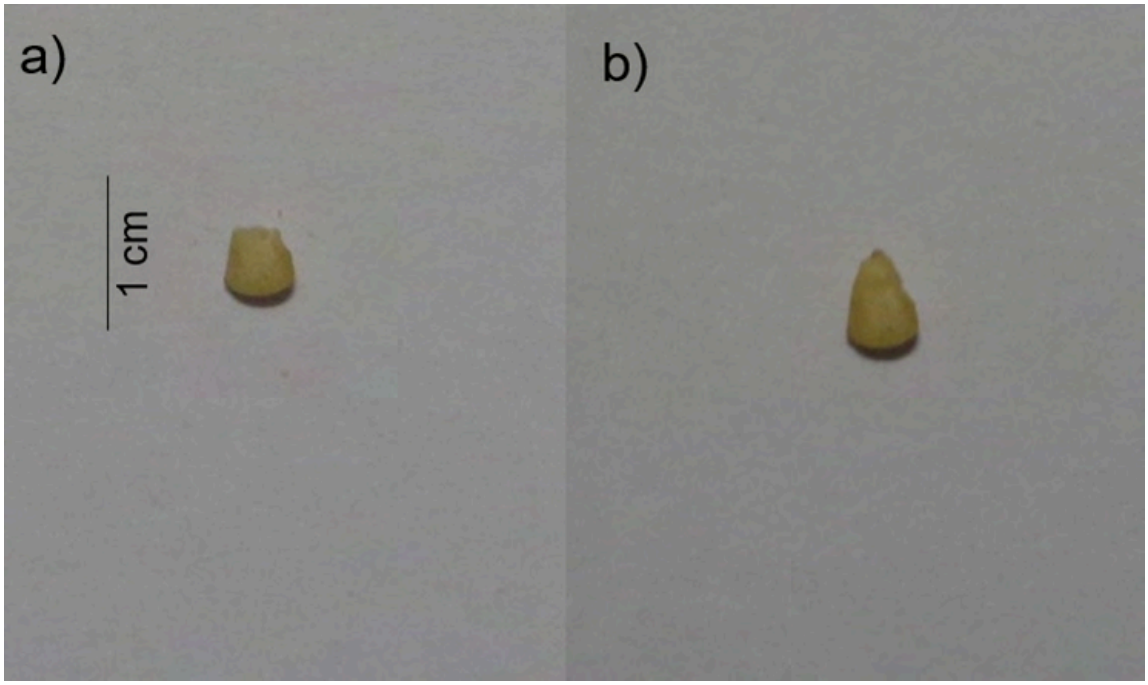
Physical scarification of seeds

The results of the physical scarification process are shown in Figure 2a for scarified guava seeds (without testa) and Figure 2b shows seeds without any scarification (with testa).

Figure 2. *Psidium guajava* L. a) scarified seed and b) whole seed.



Other research, such as that by Zurita-Valencia *et al*. (2014), during the process of scarification of seeds of *Tilia mexicana* Schlecht. (cirimo) used physical methods, such as temperature treatments, and chemical methods, such as the use of 10% hydrochloric acid (HCl), employing 70% ethanol, 3% hydrogen peroxide, and 2.4% chlorine for disinfection. Likewise, Sánchez-Soto *et al*. (2017) assure that scarification is a technique used in agriculture to have a high percentage of germination in less time.

On the other hand, for Martin (2017), the use of techniques to regenerate plants by cloning plant material for cryopreservation and conservation, among others, is essential in biotechnology to have plants available for research and their mass reproduction. From the above, it can be deduced that *in vitro* tissue culture is a tool that increases the percentage of germination through the use of seeds with techniques such as scarification for the massive propagation of species of interest and is used to increase germination and avoid chemical scarification where toxic substances such as sulfuric acid are used.

Second experiment

In vitro establishment

In the results of the Anova ($p \leq 0.05$), significant differences were detected in all treatments in the two variables recorded.

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In Tukey's means test ($p \leq 0.05$), the results of the response of the seeds to *in vitro* culture showed a lower percentage of germination and shoot length for treatment T1 (Figures 3 and 4).

Figure 3. Response of guava seeds (Tukey, $p \leq 0.05$) in the stage of *in vitro* establishment to germination (%). Means with the same letter are statistically equal, n= 10 (Tukey, $p \leq 0.05$); HSD= 3.721; T1) non-scarified seed; T2) scarified seed; T3) non-scarified seed + activated charcoal 2 g L^{-1}; T4) scarified seed + activated charcoal 2 g L^{-1}.

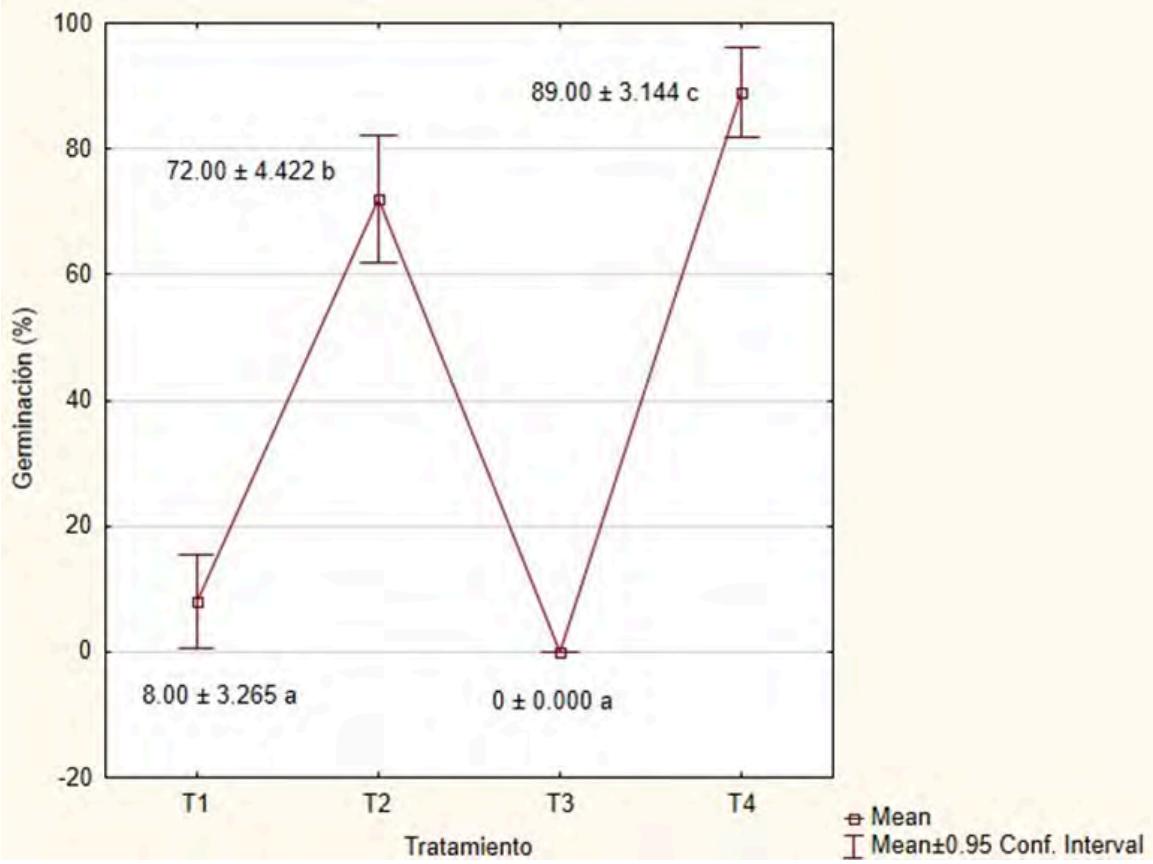

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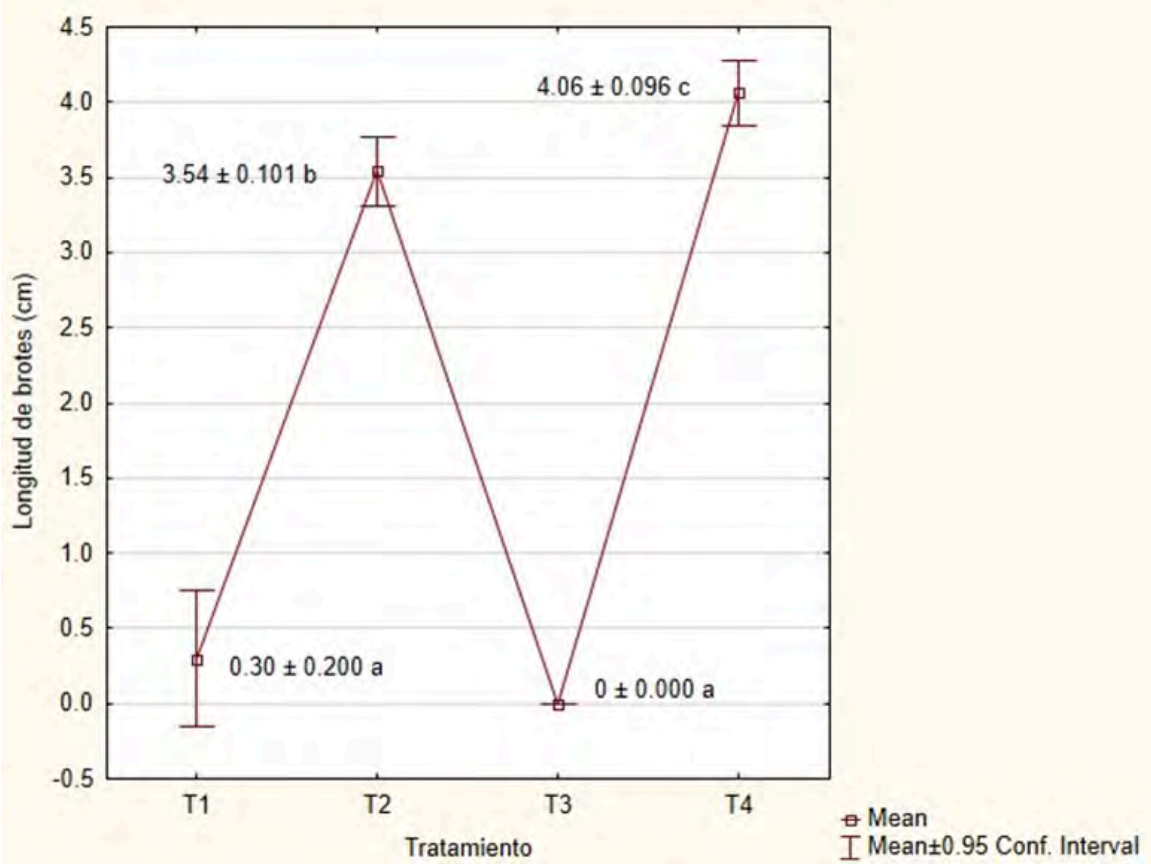
Figure 4. Response of guava seeds (Tukey, *p*≤ 0.05) in the *in vitro* establishment stage to hypocotyl shoot length (cm). Means with the same letter are statistically equal, n= 10 (Tukey, *p*≤ 0.05); HSD= 3.721; T1) non-scarified seed; T2) scarified seed; T3) non-scarified seed + activated charcoal 2 g L^{-1}; T4) scarified seed + activated charcoal 2 g L^{-1}.



In contrast to T1, T4 had the best germination and the longest shoot length. In the *in vitro* establishment stage, 90% germination was obtained with a stem length of 4.05 cm in an MS culture medium supplemented with 2 g L^{-1} of activated charcoal. Likewise, in their study of *in vitro* germination of seeds, Flores *et al*. (2017) reported 90% germination after 60 days in MS medium, without activated charcoal and in total darkness. Another very important aspect is the addition of hormones, Cabral-Miramontes *et al*. (2022) state that growth regulators and the choice of culture medium for each species serve to standardize the *in vitro* propagation methodology and play a very important role in the process, whereas Pérez *et al*. (2002) point out that the appropriate regulator for the *in vitro* multiplication of guava shoots from seeds is the BAP regulator at concentrations ranging from 0.5 to 1 mg L^{-1}. In this research, the concentration of 0.5 mg L^{-1} of BAP was used.

Finally, a standardized step-by-step protocol for aseptic establishment and *in vitro* propagation for the conservation of guava plants from scarified seeds is presented (Table 1).

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Table 1. Standardized protocol for the *in vitro* establishment of guava seeds for propagation.

	Methodology for the *in vitro* establishment of guava seeds
1	Collection of fruits to obtain seeds
2	Split the guava fruit to extract the seeds, rinse with tap water and place one by one on absorbent paper and let them dry for two days
3	The seeds are brushed one by one, rinsed with sterile water and left to dry on paper for 1 day
4	The seeds are washed with 0.1% Tween® 20 and left to stir for 10 min. Three rinses are carried out with sterile water
5	The seeds are left in stirring with Fractal® 6 ml L^{-1} for 1 h. Three rinses are carried out with sterile water
6	The seeds are placed in 5% chlorine in stirring for 15 min. Three rinses are carried out with sterile water
7	The seeds are placed in 70% ethanol in stirring for 5 min. Three rinses are carried out with sterile water
8	The seeds are left to dry on a sterile napkin in the laminar flow hood for 5 min.
9	Each seed is scarified using tweezers and a nail clipper, both sterile, in the laminar flow hood
10	Guava seeds are established *in vitro* for germination in MS culture medium supplemented with 2 g L^{-1} activated charcoal, 0.5 mg L^{-1} BAP, and 7.5 g L^{-1} agar-agar

In vitro germplasm banks aim to preserve living plant tissues for an indefinite period of time, which are kept under controlled conditions (light and temperature). Slow-growing systems have been developed to prolong subculture periods. This *in vitro* preservation technique is efficient for several species, including guava. Tissues stored under slow-growing conditions can be transferred to regeneration media and thus obtain the number of plants required to have a source of plant material, without the need to resort to collecting wild specimens, and thus help the conservation of species and native germplasm (Pérez *et al*., 2012).

Conclusions

With the development for the standardization of protocols, the foundations are established for the propagation and conservation of plant material of importance to guava producers, which allows healthy and vigorous plants to be obtained.

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Counts

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