**Short Note [Nota Corta]**

# Efficient disinfection of soursop (*Annona muricata* L.) explants for *in vitro* micropropagation †

# [Desinfección eficiente de explantes de guanábana (*Annona muricata* L.) para micropropagación *in vitro*]

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## SUMMARY

**Background**: Soursop (*Annona muricata* L.) is a tropical fruit with important nutritional and medicinal properties. Clonal multiplication of soursop by *in vitro* tissue culture is the best alternative for genetic improvement and conservation purposes. **Objective**: To reduce the level of persistent microbial contamination in soursop explants to facilitate their introduction to *in vitro* tissue culture. **Methodology**: Leaf explants (LE) and internodal segments (IS) from *ex vitro* plants were disinfected with different concentrations of active chlorine and ethanol, and different combinations of antibiotics and fungicides: Treatment 1: Streptomycin:tetracycline with carbendazim (Strept100:Prozycar); Treatment 2: Streptomycin:tetracycline with benomyl (Strept100:Benomyl); Treatments 3, 4 and 5: Same as Treatment 2, with no ethanol wash and addition of 100, 200 or 300 ppm silver nanoparticles (AgNPs), respectively. Disinfected explants were cultivated on MS medium in the presence of different combinations of plant growth regulators (2,4-dichlorophenoxyacetic acid (2,4-D), benzyladenine (BA) or kinetin (Kin). **Results**: Strept100:Prozycar and Strept100:Benomyl combinations left ~96/98 % and ~60/54 % of LE/IS contaminated, respectively. The addition of AgNPs improved decontamination (~6% of LE and ~9% of IS, respectively). Simultaneous addition of 1.5 $mg \cdot mL^{-1}$ BA and 2.0 $mg \cdot mL^{-1}$ 2,4-D, with a subsequent step with 0.5 $mg \cdot mL^{-1}$ Kin, led to the higher *in vitro* regeneration rates (~95.6% of LE and ~93.9% of IS). **Implications**: The reduction of internal contamination facilitates *in vitro* micropropagation of species of the Annonaceae family. **Conclusion**: The use of AgNPs was decisive to reduce internal contamination, improving conditions for the *in vitro* propagation of soursop tissues.
**Key words:** Plant *in vitro* regeneration; endophytic contamination; plant growth regulators.

## RESUMEN

**Antecedentes**: La guanábana (*Annona muricata* L.) es una fruta tropical con importantes propiedades nutricionales y medicinales. La multiplicación clonal de la guanábana mediante cultivo de tejidos *in vitro* es la

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mejor alternativa para fines de mejora genética y conservación. **Objetivo**: Reducir la contaminación microbiana persistente en los explantes de guanábana para mejorar su introducción al cultivo de tejidos *in vitro*. **Metodología**: Explantes foliares (LE) y segmentos internodales (IS) de plantas *ex vitro* se desinfectaron con diferentes concentraciones de cloro activo y etanol, y diferentes combinaciones de antibióticos y fungicidas: Tratamiento 1. Streptomicina:tetraciclina con carbendazim (Strept100:Prozycar); Tratamiento 2. Streptomicina:tetraciclina con benomyl (Strept100:Benomyl); Tratamiento 3. Un lavado final sin etanol, con 100, 200 o 300 ppm de nanopartículas de plata (AgNPs) (T3, T4 o T5, respectivamente), añadidas al protocolo 2. Los explantes desinfectados se cultivaron en medio MS en presencia de diferentes combinaciones de reguladores del crecimiento vegetal (ácido 2,4-diclorofenoxiacético (2,4-D), benciladenina (BA) o cinetina Kin). **Resultados**: Las combinaciones Strept100:Prozycar y Strept100:Benomyl dejaron ~96/98 % y ~60/54 % de explantes foliares/segmentos internodales contaminados, respectivamente. La adición de AgNP en el tratamiento 3 mejoró la descontaminación (~6 % de LE y ~9 % de IS permanecieron contaminados, respectivamente). La adición simultánea de 1,5 mg·mL-1 de BA y 2,0 mg·mL-1 de 2,4-D, con un paso posterior con 0,5 mg·mL-1 de Kin, dio lugar a tasas de regeneración *in vitro* más altas (aproximadamente el 95,6 % de LE y aproximadamente el 93,9 % de IS). **Implicaciones**: La reducción de la contaminación interna facilita la micropropagación *in vitro* de especies de la familia *Annonaceae*. **Conclusión**: El uso de AgNPs fue decisivo para reducir la contaminación interna, mejorando las condiciones para la propagación *in vitro* de los tejidos de guanábana.

**Palabras clave:** Regeneración de plantas *in vitro*; contaminación endofítica; reguladores del crecimiento vegetal.

## INTRODUCTION

The soursop (*Annona muricata* L.) is a tropical plant originating from Central and South America (Idowu *et al*., 2022). The major producers in the Americas are Mexico, Venezuela and Brazil, with Mexico being the leading producer worldwide (Sanusi and Bakar, 2018). In Mexico, the state of Nayarit is the leading producer (Jiménez-Zurita *et al*., 2016), with a yield of 24,718.37 t (SIAP, 2024), followed by Colima and Michoacán. Tabasco accounts for less than 0.3% of the national production but have wild-type-like soursop plantations in the Los Ríos region that have been cultivated for decades. These local-adapted varieties possess adaptative advantages against drought and flooding, as well as high resistance to pests and diseases. Since they constitute a natural source of genetic diversity, it is important to create germplasm banks for conservation and propagation purposes.

Soursop plants are mostly propagated by seeds, though air layering or grafting. However, these methods are slow and costly (Ferreira *et al*., 2019). Conversely, *in vitro* tissue culture methods are the most effective way of obtaining morphologically and genetically stable populations. Few *in vitro* tissue culture protocols for Annona species have been reported for *A. muricata* (Abubacker and Deepalakshmi, 2017, Ba *et al*., 2021), *A. squamosa* (Choudhary *et al*., 2019), *A. cherimola* Mill. (Rasai *et al*., 1995) and *A. reticulata* L. (Kudikala *et al*., 2020), using seeds, leaf explants, internodal segments, apices, as well as cotyledonal and axillary nodes, as explant sources. Seeds and explants were sterilized using antibiotics, fungicides, diluted sodium hypochlorite solutions, and toxic substances, such as mercury chloride (Abubacker and Deepalakshmi, 2017).

Previous *in vitro* regeneration studies of *A. muricata* showed low regeneration results, and consistently high contamination rates. Maldonado *et al*. (2001) reported percentages of fungal contamination of *A. muricata* segmental nodes ranging from 14% and 81.91%, and found an inverse relationship with tissue oxidation, suggesting that the decontamination methods reduce the viability of explants. Ramírez-Villalobos *et al*. (2002) reported high levels of bacterial (78%) and fungal (56%) contamination, low viability levels for soursop apices and nodal segments and did not report regeneration efficiencies. Abubacker and Deepalakshmi (2017), reported the establishment of an *in vitro* protocol for direct plantlet regeneration from internodal explants of *A. muricata*. However, they use $HgCl_2$ as decontaminating agent, a very toxic compound, and did not report decontamination efficiency.

Despite the relative success in reducing surface microbial contamination, tissue oxidation caused by the chemicals used, and residual contamination by fungi, bacteria and yeasts of endophytic origin, remain critical issues for the *in vitro* cultivation of plant material (Singh, 2018). The use of silver nanoparticles (AgNPs) as a replacement for toxic chemicals to eliminate internal and external contaminations has been investigated. Arab *et al*. (2014) found that adding different concentrations of AgNPs to the culture medium significantly

reduced fungal and bacterial contamination. However, concentrations above 150 ppm affected the viability of Garnem hybrid explants used as peach rootstock. A comparison of AgNPs and $HgCl_2$ decontamination effectiveness during strawberry micropropagation demonstrated that 200 ppm AgNPs was more effective than 1 ppm $HgCl_2$ in reducing endogenous contamination (Tung *et al*., 2021).

Based on these facts, the objective of the present study was to develop a protocol to reduce endophytic contamination in soursop explants of soursop native varieties grown in the Los Ríos region, by evaluating silver nanoparticles as a decontaminating agent.

## MATERIALS AND METHODS

### Plant materials

Ripe fruits weighing 5.2 ± 0.4 kg were collected from a plantation in Ejido Balancán in the municipality of Balancán in the state of Tabasco (latitude: 17.795799, longitude: -91.520207), 150 ± 30 days post anthesis. Three groups of ten seeds randomly selected from ten fruits were germinated in one-liter polystyrene pots containing Cosmopeat®. After 30 days, seedlings with 8-10 true leaves were transplanted into ten-liter pots containing a 1:1 mixture of Cosmopeat® and soil. The plants were maintained as an *ex situ* collection of native soursop plants under greenhouse conditions at the National Institute of Technology of Mexico, Los Ríos campus (Figure 1a, 1b, 1c).

### Disinfection of plant tissues

The fourth true leaf of donor plants was used to obtain leaf explants (LE), and the area between the third and fourth internodes was used to obtain internodal segments (IS). The LE and IS explants were thoroughly washed with sterile distilled water and then disinfected in a laminar flow hood using one of three treatments methods described in Table 1, which combined antibiotics and antifungals. The explants were immersed in the corresponding solutions in sterile Erlenmeyer flasks and shaken at 147 rpm for 20 minutes and rinsed with sterile distilled water after each immersion. The optimal concentration of AgNPs required for the decontamination of explants was determined by incubating leaf explants and nodal segments in different concentrations of AgNPs (100, 150, and 200 ppm) in Treatments 3, 4 and 5, respectively. Decontamination efficiency was evaluated by quantification of infected explants after 20 days.

### *In vitro* culture of leaf explants and internodal segments

Disinfected LE were grown in Magenta® boxes containing MS½ culture medium [MS salts (Murashige and Skoog, 1962) at half ionic strength, 30 $g \cdot L^{-1}$ sucrose, 2.3 $g \cdot L^{-1}$ Gelrite®, pH adjusted to 5.7], supplemented with the following PGR combinations: Protocol A, 1.5 $mg \cdot L^{-1}$ BA; Protocol B, 2.0 $mg \cdot L^{-1}$ 2,4-D ; Protocol C, 1.5 $mg \cdot L^{-1}$ BA and 2.0 $mg \cdot L^{-1}$ 2,4-D. The results were evaluated after 25 days in culture (DIC). In protocols D, E, and F, LE from protocols A, B and C were subcultured at 15 DIC into a MS½ medium supplemented with 1.5 $mg \cdot L^{-1}$ BA and 0.5 $mg \cdot L^{-1}$ Kin; they were then cultured for an additional 10 days, for a total of 25 DIC. Magenta® boxes were incubated in the dark in a growth room at a temperature of 25 ± 1 °C. The IS were cultured in MS½ medium in the absence of plant growth regulators (PGR) and incubated for 25 days in a growth room at 25 ± 1 °C in the dark.


**Figure 1. *Ex vitro Annona muricata* plantlets. a) Collected fruits, b) Seed germination, c) Plantlets with 8-10 true leaves nursed under growth-room conditions.**

**Table 1. Disinfection protocol for leaf explants and nodal segments of *Annona muricata* L.**

| Stage | Treatment 1 | | Treatment 2 | | Treatment 3 | | Treatment 4 | | Treatment 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Strept 100 | 1% | Strept 100 | 1% | Strept 100 | 1% | Strept 100 | 1% | Strept 100 | 1% |
| | Prozycar | 1% | Benomyl | 1% | Benomyl | 1% | Benomyl | 1% | Benomyl | 1% |
| | Silweet-70 | 0.05% | Silweet-70 | 0.05% | Silweet-70 | 0.05% | Silweet-70 | 0.05% | Silweet-70 | 0.05% |
| 2 | NaClO | 30% | NaClO | 20% | NaClO | 20% | NaClO | 20% | NaClO | 20% |
| 3 | Ethanol | 70% | Ethanol | 40% | AgNPs | 100* | AgNPs | 200* | AgNPs | 300* |

**Statistical Analysis**

The experiment was conducted using a completely randomized design in a factorial arrangement with three replicates, using five explants per experimental unit. Analysis of variance was performed on the obtained data, and the means were compared using the Tukey test ($p \leq 0.05$), with SIGMA Plot 16 (2004). Results were expressed as mean ± SD.

## RESULTS AND DISCUSSION

LE and IS subjected to sterilization Treatment 1 exhibited high levels of contamination after five DIC (approximately 96% and 98%, respectively), apparently caused by fungi, bacteria, and/or yeasts (Figure 2, T1). Both LE and IS exhibited high phenolization/necrosis (960.66 and 79±0.76, respectively) (Table 2). Tissue oxidation may be influenced by exposure to high concentrations of chlorine and ethanol, and prolonged washing times (Taghizadeh and Dastjerdi, 2020). Thus, a change of fungicide and the reduction of ethanol and chlorine concentrations were introduced (Treatment 2). These modifications led to a modest decrease of contamination in LE (60±1.07) and IS (54±0.70), but phenolization in IS was increased (91±0.9) (Figure 2, T2).

It has been demonstrated that silver nanoparticles (AgNP) eliminate or minimize the growth of internal fungal and bacterial contaminations that are difficult to kill by the surface sterilization process in woody plant explants (Hamad *et al*., 2020). Different concentrations of AgNPs were evaluated in Treatments 3, 4 and 5 (100, 200 and 300 ppm). As can be seen in panel T3 of Figure 2, a concentration of 100 ppm of AgNPs was insufficient to eliminate microbial contamination. On the other hand, contamination was reduced to 6±0.58% in LE and 9±0.9% in IS, with Treatment 4, and to 5.2±0.65% in LE and 8±1.2% in IS with Treatment 5 (Figure 2, T4; Table 2). Necrosis and phenolization were also reduced to 10.1±0.63% in LE and 8±0.93% in IS with Treatment 4, and to 10±0.81% in LE and 9±0.61% in IS with Treatment 5 (Figure 2, T5; Table 2). Based on these results, subsequent *in vitro* differentiation analyses were performed using explants that had been disinfected with 200 ppm AgNPs, to avoid the possible hormetic effects previously observed with AgNPs concentrations above 200 ppm. Reports on the use of AgNPs showed controversial effects on germination, growth and development (Siddiqi *et al*., 2022). It was reported that the addition of AgNPs significantly enhanced shoot induction rates and the proliferation and maturation of somatic embryos derived from internode explants of *Passiflora edulis* (Phong *et al*., 2023). Also, the addition of AgNPs increased the number of somatic embryos of *Panax vietnamensis* plantlets (Manh Cuong *et al*., 2021). However, the use of AgNPs reduced contamination in *Aldrovanda vesiculosa* explants, but it decreased the regeneration rate and slowed down the growth and development (Parzymies, 2021). Results demonstrated that use of AgNPs reduced contamination of tissue explants obtained from *ex vitro* soursop plants and did not affect their regenerative capacity. Our results are in accordance with reports showing that the use of AgNPs effectively reduced the contaminant levels during the *in vitro* establishment of the woody plant *Psidium friedrichsthalianum* and improved the morphology in the multiplication phase of the treated plants (Andujar *et al*., 2020).

*In vitro* regeneration of explants sterilized with Treatment 4 was induced by incubating them in MS½ medium with (LE) or without PGR (IS). LE that were initially incubated in the presence of 2.0 $mg \cdot L^{-1}$ 2,4-D or 1.5 $mg \cdot L^{-1}$ BA, or the combination of both (Figure 3a, 3b, 3c, respectively), developed callus-like structures between 15 and 20 DIC, but displayed varying degrees of necrosis by 25 DIC; thus, a different balance of PGR was evaluated. LE from protocols A, B and C were sub-cultivated into fresh MS medium with 1.5 $mg \cdot L^{-1}$ BA + 0.5 $mg \cdot L^{-1}$ Kin, after 15 DIC (when necrosis was minimal or undetectable). As observed in Figure 2d, necrosis could be avoided using protocol D, resulting in around 98% of LE developing calli. In contrast,

necrosis did not revert using protocol F (Figure 3f). Regarding protocol E, yet LE became necrotic, around 14% of the LE developed globular somatic embryo-like structures (Figure 3e).

With respect to regeneration of IS that were disinfected with Treatment 4, regeneration under the Protocol G led to a shoot formation percentage of 31% (Table 3), with at least three shoots per IS (Figure 4).

**Table 2. Disinfection efficiencies of *Annona muricata* L. leaf explants and internodal segments.**

| Composition/ Disinfection treatment | Contaminated tissues | % contamination | Necrosis / phenolization | % Necrosis / phenolization |
|---|---|---|---|---|
| Leaf explants (25) | | | | |
| Control | 25 | 100 | - - - - | - - - - |
| T1 | 24±0.5[a] | 96±0.54 | 24 ± 0.7[a] | 96±0.66 |
| T2 | 15±1.07[b] | 60±1.07 | 23 ± 0.3[b] | 93.5±0.32 |
| T3 | 14±0.8[b] | 56±1.91 | 18±1.6[c] | 72±1.64 |
| T4 | 1.5±0.6[c] | 6±0.58 | 2.5 ± 0.6[d] | 10.1±0.63 |
| T5 | 1.3±0.9[c] | 5.2±0.65 | 2.6±0.8[d] | 10±0.81 |
| Internodal segments (33) | | | | |
| Control | 33 | 100 | - - - - | - - - - |
| T1 | 32.34±2[a] | 98±1.9 | 26 ± 0.8[a] | 79±0.76 |
| T2 | 17.82±0.7[b] | 54±0.70 | 30 ± 0.9[b] | 91±0.92 |
| T3 | 18.14±0.5[b] | 55±0.5 | 24±1.2[c] | 72±1.21 |
| T4 | 2.97±0.9[c] | 9±0.9 | 2.6 ± 0.9[d] | 8±0.93 |
| T5 | 2.61±0.4[c] | 8±1.2 | 3±0.6[d] | 9±0.61 |

Values are means ± SD. Means with the same letter in each column show no significant difference, according to Tukey's test ($p < 0.05$).

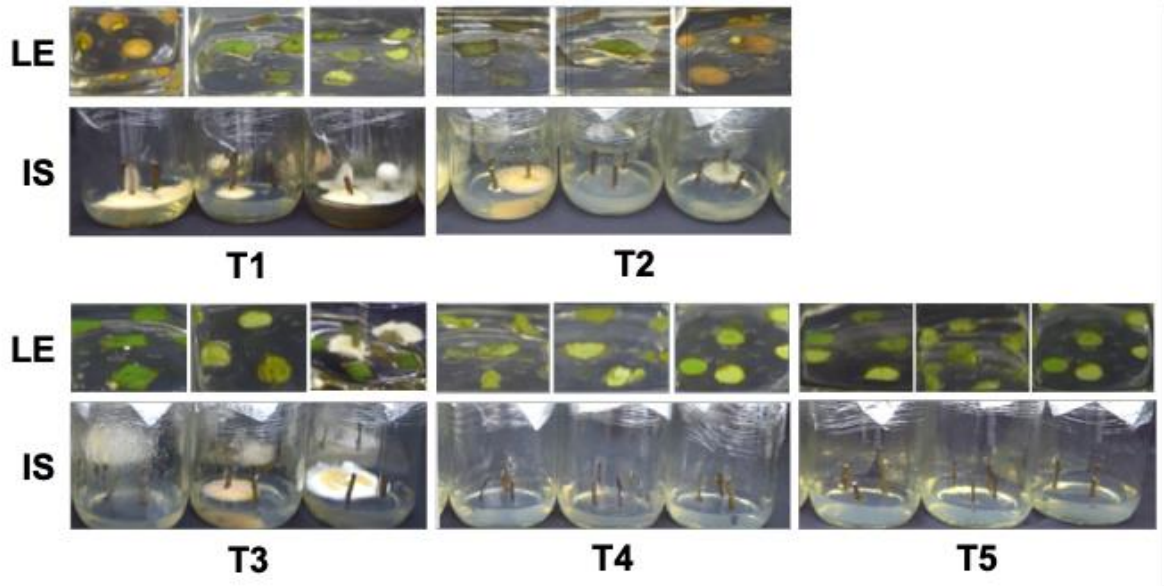


**Figure 2. Disinfection protocols of *Annona muricata* tissue explants. Leaf Explants (LE) and internodal segments (IF) were subjected to successive washes with different concentrations of active chlorine, ethanol and: Treatment 1, Streptomycin:tetracycline with carbendazim (Strept100:Prozycar) (T1); Treatment 2, Streptomycin:tetracycline with benomyl (Strept100:Benomyl) (T2); Treatment 3, Same as Treatment 2 with addition of 100 ppm silver nanoparticles (T3); Treatment 4, Same as Treatment 2 with addition of 200 ppm silver nanoparticles (T4); Treatment 5, Same as Treatment 2 with addition of 300 ppm silver nanoparticles. Treatments 3, 4 and 5 received no ethanol washes. The experiments were replicated three times and images are representative of the statistical results.**

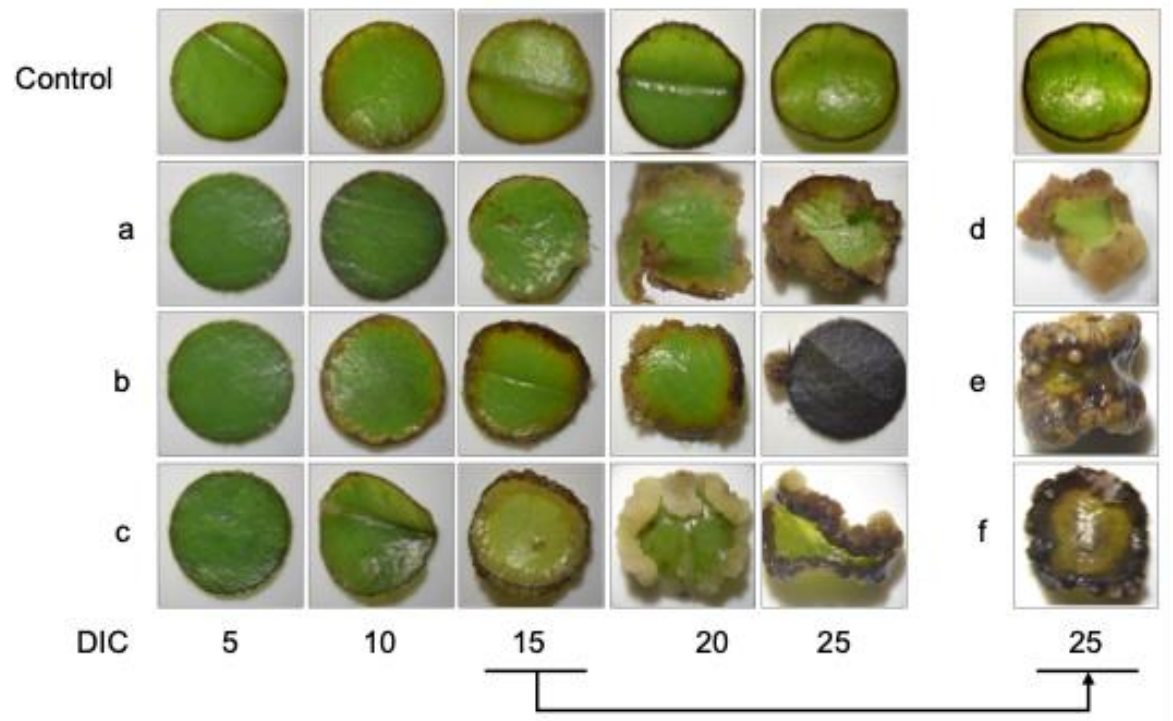


**Figure 3. Morphogenic response of leaf explants from *Annona muricata* L. Leaf explants were cultivated in MS ½ medium in the presence of different combinations of PGR: a) 2,4-D, b) BA, c) BA + 2,4-D, d) 2,4-D for 15 DIC, then BA + KIN; e) BA for 15 DIC, then BA + KIN; f) BA + 2,4-D for 15 DIC, then BA + Kin. The generation of calli/embryo-like structures was measured at different periods. The experiments were replicated three times and images are representative of the statistical results.**

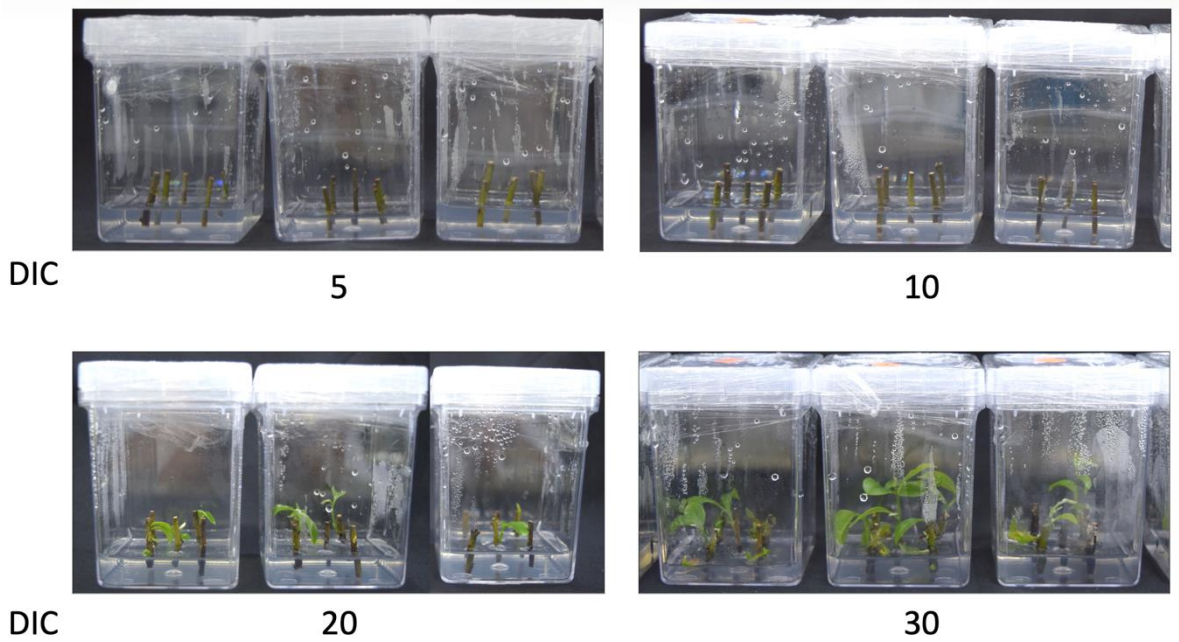


**Figure 4. Morphogenic response of internodal segments from *Annona muricata* L. internodal segments were cultivated in MS ½ medium in the absence of PGR, and the generation of new shoots was measured. The experiments were replicated three times and images are representative of the statistical results.**

**Table 3. *In vitro* regeneration efficiencies from leaf explants and internodal segments of *Annona muricata* L.**

| Regeneration protocol | Leaf Explants with development of calli* (%) | Internode Segments with new shoots** (%) |
|---|---|---|
| A | 0.0 ± 0.0 | NA |
| B | 0.0 ± 0.0 | NA |
| C | 0.0 ± 0.0 | NA |
| D | 98±1.9 | NA |
| E | 14±0.54 | NA |
| F | 0.0 ± 0.0 | NA |
| G | NA | 31±0.5 |

*Leaf explants developing calli at 25 DIC, in the presence of BA + Kin.
**Internodal segments were cultivated in MS½ in the absence of PGR.

## CONCLUSIONS

In the present work, the use of 200 ppm AgNP reduced internal contamination of leaf explants and internodal segments of *Anonna muricata* L., without apparent negative effects on their *in vitro* regeneration capacity. Results also demonstrated that good *in vitro* regeneration rates can be obtained by evaluating different combinations of PGR. The sterilization protocol eliminates the use of toxic chemical compounds and facilitates the optimization of *in vitro* multiplication protocols for soursop plants and the establishment of *in vitro* germplasm banks of soursop varieties and cultivars with outstanding agronomic traits.

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**Conflict of interest.** The authors declare that they have no conflict of interest.

**Compliance with ethical standards.** Due to the nature of the work, approval by a (bio)ethical committee is not requiered.

**Data availability.** Data are available with Daniel Pérez-Pascual (daperezp@ipn.mx).

**Author contribution statement (CRediT). D. Pérez-Pascual –** Investigation, Methodology, Funding acquisition, Writing–original draft. **A.C. Juárez-González** – Investigation, Methodology, Writing – review. **D. Jiménez-Guillen –** Writing – review, Supervision, Resources. **J.J. Zúñiga-Aguilar –** Writing – original draft – review and editing**, S**upervision, Resources.

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