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Propagación *in vitro* de callos de morera (*Morus alba* L.) como alternativa alimenticia para larvas de gusanos de seda (*Bombyx mori*)

In vitro propagation of *Morus alba* L. calli as an alternative feed for silkworm (*Bombyx mori*) larvae

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ABSTRACT: Mulberry *Morus alba* L. is a deciduous tree which leaves yield is a feeding source with high nutrients content and digestibility. The *in vitro* culture allows the vegetal tissue proliferation from young explants and the addition of growth regulators. The subject of the present research is to promote the undifferentiation cellular in leave tissue and origin callogenesis in plants of *Morus alba*, L. var. SLP5 and SLP3 supplemented with plant growth regulators 2,4-D and TDZ and evidence the acceptability of *in vitro* cultured mulberry calli in the feeding of the first silkworm larvae stages (*Bombyx mori*). The three portions of mulberry leaves var. SLP5 and SLP3 were cultured in MS modified medium supplemented with 7.0 g L^{-1} agar, 3% sucrose, 0.4 mg L^{-1} thiamine and 100 mg L^{-1} myo-inositol with 2,4- dichlorophenoxyacetic acid (2,4-D, 1 and 3 mg L^{-1}) combine with thidiazuron (TDZ, 1 and 3 mg L^{-1}), in a completed randomized design (1:1; 1:3; 3:1 and 3:3 mg L^{-1}) with 5 replicates among two months. The SLP5 variety displayed a bigger weight 2.49±0.48 g and volume 2.83±0.86 cm^3 than SLP3 variety. The calli obtained in treatment 4 (3 mg L^{-1} of 2,4-D and 3 mg L^{-1} TDZ) displayed a bigger volume 3.39±0.80 cm^3 and weight 2.93±0.33 g ($p \leq 0.05$). In regard to the leaves sections, the explants growth calli do not show significantly differences ($p \leq 0.05$). The silkworm larvae growth was bigger for treatment 4 weight 2.66±1.87 g and length de 2.62±2.10 cm ($p \leq 0.05$). It is worth to mention that the *in vitro* cultured mulberry calli as food acceptance was similar for all treatments. In this research the *in vitro* propagation of white mulberry calli was independent from the plant variety. The calli white mulberry propagated *in vitro* were accepted in the feeding of silkworm larvae under laboratory conditions.

Key words: *Morus* sp, plant growth regulators, calli, *Bombyx mori.*

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RESUMEN: La morera *Morus alba* L. es un árbol caducifolio cuya producción de hojas es utilizada como fuente alimenticia por sus altos contenidos de nutrientes y digestibilidad. El cultivo *in vitro* permite la proliferación de tejidos vegetales a partir de explantes jóvenes y la adición de reguladores de crecimiento. El objetivo de la presente investigación es promover la desdiferenciación celular en tejido de hojas y originar callogénesis en plantas de *Morus alba*, L. var. SLP5 y SLP3 suplementado con reguladores de crecimiento 2,4-D y TDZ y evidenciar la aceptabilidad de los callos de morera cultivados *in vitro* en la alimentación de los primeros estadios larvarios de los gusanos de seda (*Bombyx mori*). Se cultivaron explantes de tres porciones de hojas de morera de var. SLP5 y SLP3 en medio de cultivo modificado de MS suplementado con 7.0 g L^{-1} agar, 3% sacarosa, 0.4 mg L^{-1} tiamina y 100 mg L^{-1} mio-inositol con 2,4- ácido diclorofenoxiacetico (2,4-D, 1 y 3 mg L^{-1}) combinado con tidiazuron (TDZ, 1 y 3 mg L^{-1}), bajo un diseño completamente al azar (1:1; 1:3; 3:1 y 3:3 mgL^{-1}) con 5 repeticiones durante dos meses. La variedad SLP5 mostró mayor peso 2.49±0.48 g y volumen 2.83±0.86 cm^3 que la variedad SLP3. Los callos obtenidos en el tratamiento 4 (3 mg L^{-1} de 2,4-D y 3 mg L^{-1} TDZ) mostraron mayor volumen 3.39±0.80 cm^3 y peso 2.93±0.33 g ($p \leq 0.05$). Respecto a las secciones de hoja, los callos crecidos a partir de los explantes no mostraron diferencias significativas ($p \leq 0.05$). El crecimiento de las larvas de gusano de seda fue mayor para el tratamiento 4 con un peso 2.66±1.87 g y longitud de 2.62±2.10 cm ($p \leq 0.05$). Cabe mencionar que la aceptación de los callos de morera producidos *in vitro* en la alimentación de larvas de gusano de seda fue similar para todos los tratamientos. En esta investigación la propagación *in vitro* de los callos de morera blanca fue independiente de la variedad de planta. Los callos de morera propagados *in vitro* fueron aceptados en la alimentación de larvas de gusano de seda bajo condiciones de laboratorio.
Palabras clave: *Morus* sp, reguladores de crecimiento en plantas, *Bombyx mori.*

INTRODUCTION

The *Morus* sp (mulberries) are flowering and deciduous plants of *Moraceae* family. Mulberries have been domesticated over thousands of years and are cultivated in Asian, European, American, and African continents (Özgen *et al*., 2009). There are many applications to mulberries, which include livestock feeding (Sánchez, 2000; Milera *et al*., 2010; Islam *et al*., 2014) gardening or landscaping (Ljubojević *et al.*, 2023), environmental safety (Mallick & Sengupta, 2022), and natural products (Özgen *et al.*, 2009; Mallick & Sengupta, 2022). Their multiple applications led to great exploitation worldwide, from smallholders to large-scale production (Sánchez, 2002). The impressive adaptability of mulberries comes from their simple cultivation, fast growth, and survival under challenging environments (Rahman & Islam, 2021).

The main agronomic application of mulberries is for leaf harvest and thus feeding the silkworm (*Bombyx mori*) under sericulture or silk farming (Özgen *et al*., 2009; Bobis *et al.*, 2018; Mallick & Sengupta, 2022). Importantly, mulberries leaves are the sole food provided to silkworms (Kumar *et al*., 2012; Rahman & Islam, 2021). Data from multiple reports indicated that fruits and leaves have high content of crude protein, sugars, fatty acids, micronutrients, and dry-matter digestibility, which supports its multiple applications (Bamikole *et al*., 2005; Liang *et al*., 2012; Koyunco *et al*., 2014; Zach *et al*., 2017). However, mulberries require adequate management practices to provide such high nutritional content (Benavides *et al*., 1994). Mulberry are deciduous trees and their leaf production is destined to feeding the silkworm larvae, however through winter and under plague and disease attack thous yield is diminished and the leaves quality reduce (Banday *et al*., 2017).

In vitro culture of plant tissues allows large-scale propagation of desirable genotypes under sterile conditions (Sharma & Thorpe, 1990; Sil, 2021). Such approaches usually rely on young explants for *in vitro* propagation, since there is greater viability (Acuña, 2020), alongside the use of plant growth regulators (Freire-Seijo, 2003; Ikeuchi *et al*., 2013; Geetha & Murugan, 2017). *In vitro* mulberry calli culture is strongly influence by the addition of plant growth regulators to the culture media. The auxin and cytokinine stimulate necessary reactions for the calli, steam or

rooths *in vitro* culture from an explant (Ikeuchi *et al*., 2013). Bhau y Wakhlu (2001) report the use of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA) (1.0 mg L^{-1} y 0.5 mg L^{-1}) successfully in the propagation of mulberry calli from leaf explants, internodes and stalk.
The *in vitro* propagation of mulberries calli offers the possibility of feeding silkworm larvae continuously. The utilization of sterile mulberry calli production as a model in silkworm larvae feeding guaranty the availability of silkworms as a research organism model in further applications (Ashraf & Qamar, 2023).
The aim of this study was to determine the effect of variety alongside plant growth regulator supplementation on i*n vitro* propagation of white mulberries (*Morus alba* L.) calli and its application as a supplementary feed for growing silkworm larvae.

MATERIAL AND METHODS

Location and vegetal material
The project was developed according to institutional and national legislation. The experiments were performed in the plant tissue culture laboratory at the Departamento de Fitotecnia, Universidad Autónoma Chapingo, located in Texcoco, Mexico in 2014.
The vegetative material was obtained from two years old white mulberry trees (*Morus alba* L.) of the SLP3 and SLP5 varieties grew and acclimated in a greenhouse. The white mulberry characteristics were SLP3 larger ≈20cm softer (pliable) and thinner leaves and SLP5 smaller ≈15cm visibly thicker and brittle leaves.
Plants were placed in 40 x 40 cm polyethylene pots filled with 3 to 6 mm tezontle substrate and were irrigated with 250 ml tap water twice a day and supplemented weekly with a 3 ml L^{-1} Byfolan®, 0.5g L^{-1} Aliette® and 1.5g L^{-1} AgrimyCu® solution by sprinkled to control contamination in plants. Plants were cultivated by 1.5 years.

In vitro growth leaves culture
The *in vitro* culture was established with 20 SLP3 and 20 SLP5 young leaves (seven to eight cm length) that were washed with Roma® detergent (5g L^{-1}) and Tween™-80 (Fisher Chemical) hi-purity (1 ml L^{-1}) in distilled water for 20 min. Further, leaves were surface disinfest with 70% ethanol for three minutes, and subsequently in a solution of 10 ml•100 ml sodium hypochlorite (Cloralex®). Leaves of mulberries were transferred to Petri dishes and rinsed three times with sterile ultra-purify water.
The culture medium to *in vitro* propagation was based on a modified version of the MS medium (Sigma Aldrich ®) (Murashige & Skoog, 1962) supplemented with 7.0 g L^{-1} Meyer ® agar (≥99%), 3% sucrose (99.5%) (Sigma Aldrich ®), 0.4 mg L^{-1} thiamine (≥99%) (Sigma Aldrich ®), and 100 mg L^{-1} myo-inositol (≥99%) (Sigma Aldrich ®). The pH of the solution was adjusted to 5.7 $\pm$ 0.1. 20 ml the culture medium was placed in (200 ml) Gerber® flasks, which were sealed with polyethylene tops, and sterilized by autoclaving at 121°C, 15 PSI for 40 min. Afterwords the flasks were set at room temperature 22 ± 1°C and the plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) (≥95%) and thidiazuron (TDZ) both from Sigma Aldrich® were added as is described in experimental design treatments.
Leaves rinsed in sterile ultra-purify water were dissected with scalpel in three portions: apical, medium and basal acording to Freschi *et al.* (2010). Each leaf portion were placed randomly in Gerber® flasks with culture medium MS for each treatment. Flasks were sealed to avoid contamination and placed in a dark growth chamber at 22±1°C, culture was performed for 60 days.

Explants decontamination protocol
The explants exposed to contamination were retrieved from *in vitro* culture and submerged in a Roma® detergent (5g L^{-1}) and Tween™-80 (Fisher Chemical) hi-purity (1ml L^{-1}) solution in distilled water. Subsequently, explants were submerged in 70% ethanol for three min, submerged in 10 ml•100 ml sodium hypochlorite (Cloralex®) solution for 15 min, and placed with 4%

calcium hypochlorite for 15 min. Additionally, explants were exposed to a 100% Timsen® solution for 15 min, Benlate® and Fungimycin 100® 2g L^{-1} solutions for 10 min, and rinsed in 100% Protect® H_2O_2 before being placed in a Petri dish containing MS medium.

Experimental Design

To determinate the effect of mulberries (*Morus Alba* L.) variety and supplementation of plant growth regulators on *in vitro* mulberry calli propagation, a completely randomized design was performed with four treatments: *i*) 1 mg L^{-1} 2,4-D/ 3 mg L^{-1} TDZ, *ii*) 3 mg L^{-1} 2,4-D/1 mg L^{-1} TDZ, *iii*) 1 mg L^{-1} 2,4-D/1 mg L^{-1} TDZ and *vi*) 3 mg L^{-1} 2,4-D/3 mg L^{-1} TDZ with five replicates; each flask was an experimental unit. There were evaluate weight and volume as non-destructive measurements (quantitative variables) and visual coloring as qualitative variable for *in vitro* grown calli, during the culture period in 14 days intervals.

Evaluation of in vitro *grown calli to feed Bombyx mori larvae*

The unhatching silkworm eggs were obtained from the Universidad Politécnica Francisco I. Madero, Tepatepec - Hidalgo, Mexico and were maintained at room temperature at 22±1°C in the plant tissue culture laboratory until larvae hatching.

Five silkworm larvae were placed in 10 cm disposable Petri dishes at room temperature at 22 ±1°C immediately after hatching. The explant *in vitro* grown and calli were used to feed the silkworm larvae totally the first 21 days, twice a day (9:00 and 19:00 h). The effect of feeding with *in vitro* grown calli of white mulberries was determined by measuring the weight and length of silkworm larvae at seven days intervals. The silkworm larvae survival percent was determined at the end of the 21 days experiment. Subsequently the larvae were feed 25 days more with mulberry leaves until the cocoon weave at day 46 and the emerging of the moth after 12 to 15 days after.

Statistical analysis

The variables weight and volume of the *in vitro* grow mulberry calli and the length and weight of the silkworm larvae feed with *in vitro* grow calli were analyzed with an analysis of variance (ANOVA) and the *post-hoc* analysis was done with Tukey's mean test at 5% significance level with the software Statistical Analysis System® (SAS) 9.0.

RESULTS AND DISCUSSION

In vitro propagation of white mulberries calli from leaves explants

The differences in development of white mulberries calli *in vitro* became evident from week two onward (**Figure 1**).

The treatment T4 and T2 displayed the best traits, which included calli weight and volume (**Table 1**) ($p \leq 0.05$). Under the comparison between varieties of white mulberries, the variety SLP5 was different in weight and volume than the variety SLP3, ($p \leq 0.05$) (**Table 2**).

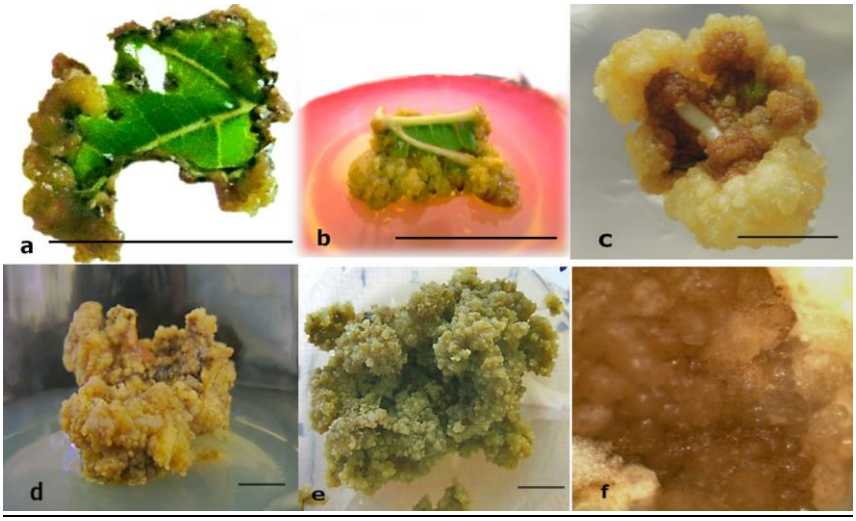

Figure 1. *In vitro* culture of *Morus alba* L. calli from leaf explants. a-c) Second, fourth, and sixth week after *in vitro* culture respectively; d-e) Eighth week; f) Enlarged view of calli during the eighth week of culture. Scale barr 0.5 cm.
Figura 1. Cultivo *in vitro* de callos de *Morus alba* L. a partir de explantos foliares. a-c) Segunda, cuarta y sexta semana después del cultivo *in vitro*, respectivamente; d-e) Octava semana; f) Vista ampliada de los callos durante la octava semana de cultivo. Barra de escala de 0.5 cm.

Table 1. Mean weight and volume *in vitro* grow calli of white mulberry (*Morus alba* L.) suplemented with 2,4-D and TDZ.
Tabla 1. Peso y volumen medio de callos de cultivo *in vitro* de morera blanca (*Morus alba* L.) suplementados con 2,4-D y TDZ.

Treatment*	Weight (g)	Volume (cm^3)
4	2.93±0.33[a]**	3.39±0.79[a]
2	2.89±0.24[a]	3.20±0.55[a]
1	2.07±0.19[b]	2.080±0.17[b]
3	2.02±0.11[b]	2.28±0.34[b]
MSD	**0.1097**	**0.2414**

*Treatments 1) 1 mgL^{-1} 2,4-D/ 3 mgL^{-1} TDZ, 2) 3 mgL^{-1} 2,4-D/1 mgL^{-1} TDZ, 3) 1 mgL^{-1} 2,4-D/1 mgL^{-1} TDZ and 4) 3 mgL^{-1} 2,4-D/3 mgL^{-1} TDZ.
**Means different superscript letters, within the same columns are statistically difference significative Tukey´s test (<0.05).

Table 2. Comparison of variety on weight and volume of *in vitro* grown calli of white mulberry (*Morus alba* L.).
Tabla 2. Comparación de variedades en peso y volumen de callos cultivados *in vitro* de morera blanca (*Morus alba* L.).

Variety	Weight (g)	Volume (cm^3)
SLP5	2.48±0.48[a*]	2.83±0.86[a]
SLP3	2.46±0.51[a]	2.68±0.67[b]
MSD	**0.0591**	**0.13**

*Means different superscript letters, within the same columns are statistically difference significative Tukey´s test (<0.05). MSD: minimum statistical difference.

However, the portions of leaves did not affect significantly *in vitro* growth (**Table 3**). The *in vitro* grown calli displayed friable texture, separately from the treatment. Further, most *in vitro* grown calli of white mulberries were spongy-like and granular. The color of *in vitro* grown calli changed during their growth (**Table 4**), gradually mulberry calli acquired a darker coloring due the natural tissue oxidation (**Figure 2**).

Table 3. Mean weight and volume of *in vitro* grown calli of white mulberry (*Morus alba* L.) obtained from three different leaf sections.
Tabla 3. Peso y volumen medio de callos cultivados *in vitro* de morera blanca (*Morus alba* L.) obtenidos de tres secciones de hojas diferentes.

Leaf part	Weight (g)	Volume (cm3)
Basal	2.52±0.19[a*]	2.72±0.39[a]
Medium	2.46±0.19[a]	2.69±0.42[a]
Apical	2.44±0.26[a]	2.79±0.56[a]
MSD	**0.0866**	**0.1906**

*Means different superscript letters, within the same columns are statistically difference significative Tukey´s test (<0.05). MSD: minimum statistical difference.

Table 4. Calli color obtained from *in vitro* culture of white mulberry (*Morus alba* L.) varieties exposed to different auxin supplementation.
Tabla 4. Color de callos obtenidos del cultivo in vitro de variedades de morera blanca (*Morus alba* L.) expuestas a diferentes suplementaciones de auxinas.

		Treatments*			
Days	**Variety**	**T1**	**T2**	**T3**	**T4**
14	SLP5	Yellow	Yellow	Yellow	Yellow
28	SLP5	Yellow	Yellow-cream	Yellow-cream	Yellow
42	SLP5	Yellow-cream	Yellow-orange	Yellow-orange	Yellow-orange
56	SLP5	Orange-coffee	Yellow-coffee	Yellow-coffee	Yellow-orange
14	SLP3	Yellow	Yellow	Yellow	Yellow
28	SLP3	Yellow	Yellow-cream	Yellow-cream	Yellow
42	SLP3	Yellow-cream	Yellow-orange	Yellow-orange	Yellow-orange
56	SLP3	Orange-coffee	Yellow-coffee	Yellow-coffee	Yellow-orange

*T1: 1mgL^{-1} 2,4-D/ 3mgL^{-1} TDZ; T2: 3mgL^{-1} 2,4-D/1mgL^{-1} TDZ; T3: 1mgL^{-1} 2,4-D/1mgL^{-1} TDZ and T4: 3mgL^{-1} 2,4-D/3mgL^{-1} TDZ.

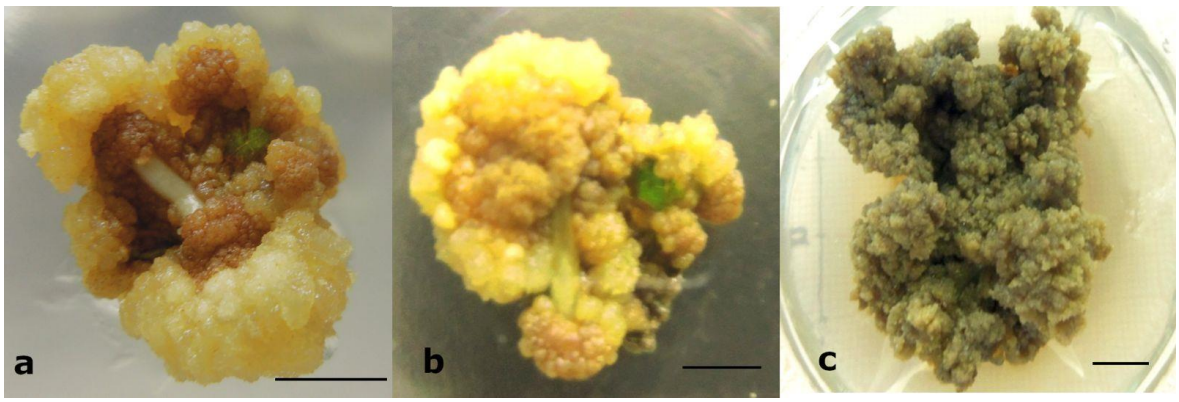

Figure 2. Color of *Morus alba* L. calli under *in vitro* conditions. a) Fourth week of culture. b) Seventh week of culture. c) Necrosis in calli at the eighth week of culture. Scale barr 1 cm.
Figura 2. Color de los callos de *Morus alba* L. en condiciones *in vitro*. a) Cuarta semana de cultivo. b) Séptima semana de cultivo. c) Necrosis en los callos en la octava semana de cultivo. Barra de escala de 1 cm.

Plant growth regulators supplementation with 2,4-D was the best approach for growing *in vitro* calli of white mulberries. The culture conditions are paramount for plant regeneration during *in vitro* propagation (Taha *et al*., 2020), while much progress was made in this technology for

mulberries (Thomas, 2002; Vijayan *et al*., 2011; Taha *et al*., 2020). Plant growth regulators are instrumental to calli induction and plant regeneration under *in vitro* conditions (Freire-Seijo, 2003; Geetha & Murugan, 2017). Ikeuchi *et al.* (2013) mention that the auxin and cytokinine stimulate necessary reactions for the calli, steam and roots *in vitro* cultured from an explant. Several growth regulators (e.g., auxins, cytokinins) have been tested for *in vitro* propagation of mulberries (Geetha & Murugan, 2017; Fonseca-Carrasco *et al*., 2020; Taha *et al*., 2020). The most prominent role of 2,4-D was similar to several previous studies (Pierik, 1990; Bhau & Wakhlu, 2001; Cholo & Delgado, 2011; Espinosa *et al*., 2012), thus demonstrating its prominent role of the growth and development of mulberries.

During experiment the grow *in vitro* explants get contaminated with fungi and bacteria from 120 flasks get contamined 13 flasks (11%) and was recuperated 10 flasks (80%) by the decontamination protocol. In Hernández & González (2010), mentioned that the season of the year and the collection procedure are a frequently origin of contamination and made emphasis in the need of developing decontamination protocols for *in vitro* culture plant tissues.

Feeding silkworm larvae with white mulberries calli

The feeding silkworm larvae with *in vitro* grown white mulberry calli with different concentration of plant growth regulator affect the larvae growth, denote a bigger weight and length in T4 (**Table 5**).

Table 5. Mean length and weight of silkworm (*Bombyx mori*) larvae fed with *in vitro* grown calli of white mulberry (*Morus alba* L.) for 21 days

Tabla 5. Longitud y peso promedio de larvas de gusano de seda (*Bombyx mori*) alimentadas con callos de morera blanca (*Morus alba* L.) cultivados in vitro durante 21 días

Treatment*	Lenght (cm)	Weight (g)
4	2.62±2.10^{a}**	2.66±1.87^{a}
2	2.52±2.04^{ab}	2.60±1.85^{a}
3	2.47±2.00^{b}	2.59±1.85^{a}
1	2.35±1.92^{c}	2.46±1.77^{b}
MSD	**0.1020**	**0.0771**

*Treatments 1) 1 mgL^{-1} 2,4-D/ 3 mgL^{-1} TDZ, 2) 3 mgL^{-1} 2,4-D/1 mgL^{-1} TDZ, 3) 1 mgL^{-1} 2,4-D/1 mgL^{-1} TDZ and 4) 3 mgL^{-1} 2,4-D/3 mgL^{-1} TDZ.

**Means different superscript letters, within the same columns are statistically difference significative Tukey´s test (<0.05).

The silkworm larvae stage has a variable duration that depends from the environmental conditions; at 20 °C the cycle length is about 50 to 55 days (Rodríguez *et al*., 2012), in this research the larval stage showed a 45 days duration in normal conditions at room temperature at 22±1°C (**Figure 3**). The survival of silkworm larvae was 57.14%, from this, 23 larvae, 100% weaved silk cocoons emerge into moths. Silk cocoons had a mean length of 2.54 ±0.20 cm and a mean diameter of 1.315 ± 0.09 cm (**Figure 4**).

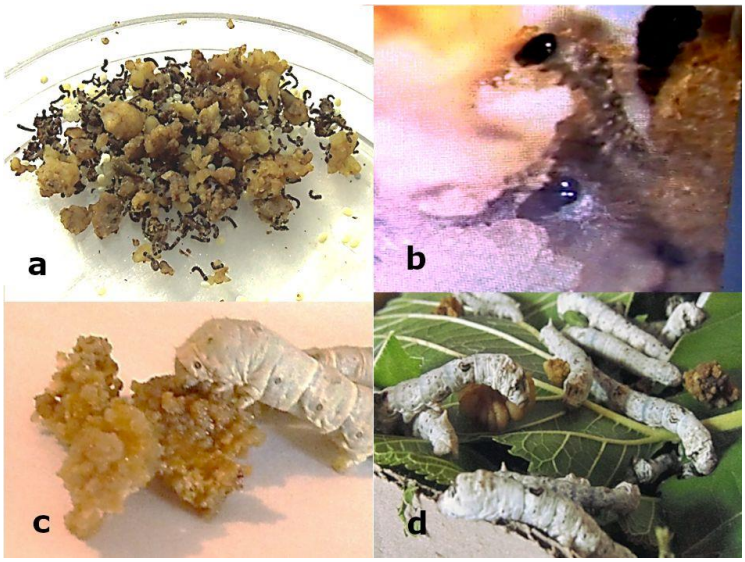

Figure 3. Feeding silkworm (*Bombyx mori*) larvae with *in vitro* grown calli of *Morus alba* L. a -b) First larval stage. c-d) Fourth larval stage.
Figura 3. Alimentación de larvas de gusano de seda (*Bombyx mori*) con callos cultivados *in vitro* de *Morus alba* L. a-b) Primer estadio larvario. c-d) Cuarto estadio larvario.

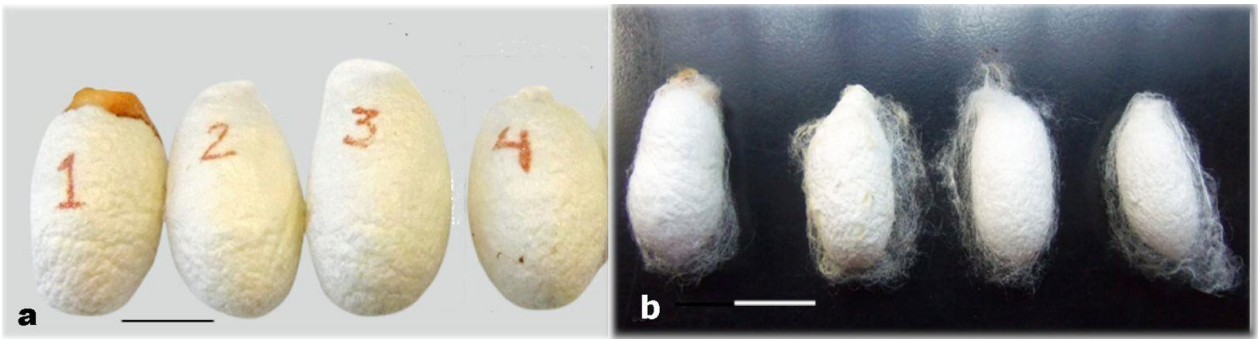

Figure 4. Cocoons obtained from adult silkworms (*Bombyx mori*) fed for 35 days with *in vitro* grown calli of *Morus alba L.* varieties SLP5 (a) and SLP3 (b). Scale barr 1 cm.
Figura 4. Capullos obtenidos de gusanos de seda adultos (*Bombyx mori*) alimentados durante 35 días con callos cultivados *in vitro* de *Morus alba* L., variedades SLP5 (a) y SLP3 (b). Barra de escala de 1 cm.

The fact that silkworm feeding relies solely on mulberries makes it susceptible to undersupply if the plant faces environmental challenges or incidence of disease and blight (Kumar *et al*., 2012; Caccam & Mendoza, 2015; Rahman & Islam, 2021). Therefore, technology alternatives that safeguard the feeding supply for silkworms is highly desirable. As far as is known, the *in vitro* grown mulberries have not been tested for feeding silkworms. Under such scenario, in this research the *in vitro* propagation of mulberries calli were feeding acceptable by silkworm larvae under sterile conditions (Sharma & Thorpe, 1990; Sil, 2021).

The white mulberry SLP5 variety was the most productive between those tested here for *in vitro* growth of calli. There is vast evidence that mulberry variety plays an important role on *in vitro* propagation efficiency (Bhatnagar *et al*., 2001; Taha *et al*., 2020). These results were similar to previous reports on the growth rate of plants (Rodríguez-Ortega *et al*., 2013), thus suggesting that *in vitro* growth may mirror the *in vivo* growth kinetics among varieties.

CONCLUSION

In this research the *in vitro* propagation of white mulberry was independent on plant variety. White mulberry *in vitro* growth calli under treatment four and two ($3mgL^{-1}$ 2,4-D/$3mgL^{-1}$ TDZ *and* $3mgL^{-1}$ 2,4-D/$1mgL^{-1}$ TDZ) performed the best characteristics in the evaluated variables. The *in vitro* propagation of mulberries calli were acceptable as food by silkworm larvae under laboratory conditions.

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