Scientific Paper

In vitro multiplication of *Morus alba* L. Criolla variety in temporary immersion systems

Jorge Liusvert Pérez-Pérez[1] *https://orcid.org/0000-0003-3372-7559*, Maylín Fonseca-Yero[2] *https://orcid.org/0000-0002-9566-0751*, Marisel Bahi-Arevich[1] *https://orcid.org/0000-0001-6822-2492*, Juan José Silva-Pupo[1] *https://orcid.org/0000-0003-1049-5740* and Stefaan Werbrouck[3] *https://orcid.org/0000-0001-5195-7054*

[1] Centro de Estudios de Biotecnología Vegetal, Universidad de Granma. Carretera a Manzanillo, km 17,5 Peralejo, CP 85100, Bayamo, Granma, Cuba. [2] Facultad de Ciencias Agropecuarias, Universidad de Granma. Carretera a Manzanillo, km 17,5 Peralejo, CP 85100, Bayamo, Granma, Cuba. [3] Laboratory of Applied In Vitro Plant Biotechnology. Ghent University. Campus Schoonmeersen C4.52 Valentin Vaerwyckweg 1, 9000 Ghent, Belgium. E-mail: jperez@udg.co.cu

Abstract

Objective: To evaluate the *in vitro* multiplication of *Morus alba* L., Criolla variety, in temporary immersion systems (SETIS™).

Materials and Methods: Two trials were conducted for the evaluation of three immersion times (1,0; 3,0; 5,0 min.) and three immersion frequencies every four, six and eight hours. Afterwards, another experiment was conducted for the analysis of the morphological response of the sprouts, at 28, 45 and 60 days of cultivation. A complete randomized design was used, with three repetitions in all the experiments.

Results: The results related to the immersion time showed significant differences in the evaluated variables for $p < 0,05$, except in the number of sprouts. In addition, it was found that the highest number of axillary buds was obtained in the immersion time of three minutes, with a value of 4,67. Meanwhile, in the length of the sprouts, the maximum value was achieved with three minutes of immersion (3,0 cm) with significant differences ($p < 0,05$) with regards to the other treatments. With the use of temporary immersion systems, with three minutes of immersion every eight hours, during 45 days of cultivation, the highest number of axillary buds and 1,90 sprouts were obtained; while the sprout length reached 9,82 cm.

Conclusions: In the sprouts no morphophysiological changes were observed, ascribed to the cultivation conditions. It was proven that the *in vitro* micropropagation of mulberry, Criolla variety, in temporary immersion systems SETIS™, is possible.

Keywords: bioreactors, tissue culture, *Morus alba*, plant growth regulators

Introduction

Morus alba L. (mulberry) is a forage plant that originated in the Himalayas, which has shown excellent qualities for feeding different animal species, whose nutritional value is one of the highest among non-legume tropical forages (Martín *et al*., 2017). In addition, it constitutes the exclusive source for feeding the silkworm (*Bombyx mori* L.), containing unique biochemical compounds in its leaves, such as morin and beta-sitosterol, which grant it an exceptional role in silk biosynthesis (Sarkar *et al*., 2018).

In some genotypes, propagation by seed is possible, but the main way is through the use of stakes (Wani *et al*., 2019). Although the latter guarantees higher homogeneity of the plantations, requires from six to seven months of maturity to make the cuts in the donor plants, and its use limits the feed availability for the animals (Vijayan *et al*., 2014). Besides, it reduces the vigor of plants in the next generations, with lower development of the root system, which leads to a reduced adaptability of daughter plants and restricts the cultivation of varieties specific from each region (Wani *et al*., 2019).

With the current biotechnological technologies it is possible to obtain plants *in vitro* in liquid culture media, considered more effective than the semisolid ones, allowing the plants higher accessibility to the components of the culture media and higher gain in biomass. The propagation time is also reduced, scaling up and its automation is possible, and no gelifiers are required, which decreases production costs in commercial laboratories (Carvalho *et al*., 2019).

Among the technologies that use liquid culture media are temporary immersion systems (TIS), semi-automated platforms that allow during a brief period the controlled contact of the material to be propagated with a liquid medium in an aseptic environment (Georgiev *et al*., 2014). In some species, the physiological response of the plants has been improved and the multiplication indexes have

Received: October 31, 2019
Accepted: September 08, 2020

How to cite this paper: Pérez-Pérez, J. L.; Fonseca-Yero, Maylín; Bahi-Arevich, Marisel; Silva-Pupo, J. J. & Werbrouck, S. *In vitro* multiplication of *Morus alba* L. Criolla variety in temporary immersion systems. *Pastos y Forrajes*. 43 (3):221-229, 2020.

been increased (Businge *et al*., 2017; Sarkar *et al*., 2018; Gianguzzi *et al*., 2019).

Although the *in vitro* regeneration of *M. alba* has been described by diverse authors (Salas *et al*., 2005; Gogoi *et al*., 2017), in this species the information about the use of methodologies with the utilization of temporary immersion systems, which allow scaling up at commercial level, is limited. Until the present, there is the antecedent of the work conducted by Salas *et al*. (2011), at the Plant Biotechnology Institute (IBP, for its initials in Spanish) of Santa Clara, Cuba. These authors used a bioreactor of temporary immersion (BIT®), constituted by two twin containers, connected between them by a silicon hose.

Today there is a commercial variant, known as TIS, called SETIS™ (Vervit, 2016). It is a modification of the systems based on flow and reflow, which constitutes a simplification of the system of twin flasks (Georgiev *et al*., 2014). It is composed by two horizontal polypropylene rectangular-shaped recipients, coupled one over the other, which allows higher utilization of the surface (Vervit, 2016). This type of system has proven to be an efficacious tool for the micropropagation of different economically important plant species from the genera *Saccharum*, *Musa*, *Solanum*, *Dioscorea*, *Phalenopsis*, *Stevia* and of forestry species such as *Eucaliptus* and *Paulownia,* among others (Balogun *et al*., 2017; Rosales *et al*., 2018).

Nevertheless, the non-optimum cultivation conditions in TIS can lead to the formation of hyperhydric plants. This type of plant develops a degree of morphoanatomic and physiological disorder that affects its regeneration capacity, *in vitro* multiplication and survival under certain environmental conditions (Quiala, 2012). This requires the study of the main indicators that influence the micropropagation and its optimization in each genotype, to achieve its effective use at commercial scale.

From the above-mentioned elements, the objective of this work was to evaluate the *in vitro* multiplication of *M. alba*, Criolla variety, in temporary immersion systems SETIS™.

Materials and Methods

Location. The research was conducted at the Center of Studies on Plant Biotechnology (CEBVEG) of the University of Granma, between December, 2018, and May, 2019.

Plant material. Nodal segments of 1,0 cm length, with one axillary bud, were taken from *in vitro* plants in second multiplication subculture, in semisolid culture medium of mulberry, Criolla variety, according to the methodology proposed by Salas *et al*. (2005).

Culture medium. For the multiplication in temporary immersion systems, the culture medium proposed by Sales *et al*. (2011) was used. For such purpose, the whole salts were used, including vitamins in Murashige and Skoog medium (Murashige and Skoog, 1962) 4,40 g L^{-1} (Duchefa Biochemie B.V.), which contains myo-inositol 100 mg L^{-1} and glycine 2,0 mg L^{-1}; besides sucrose 30 g L^{-1}, 6-benzylaminopurine (6-BAP) 0,5 mg L^{-1} and naphthaleneacetic acid (NAA) 0,5 mg L^{-1}. The pH was adjusted to 5,8 with NaOH (0,1N) and HCl (0,1N) solutions, before sterilization.

The silicon hose, hydrophobic filters (0,22 µm, MIDISART), SETIS™ flasks and culture medium, were sterilized in vertical autoclave (BK75) at 121 ºC and 1,2 kg cm^2 of pressure during 20 min. Afterwards, in the laminar flow cabinet Vitrofural® (116 mg L^{-1}) was added when the culture medium had an approximate temperature of 80-90 ºC. At the end, it was agitated until achieving homogenization.

Cultivation conditions. The cultivated explants in temporary immersion systems SETIS™, were placed in growth chamber with indirect sunlight (Salas *et al*., 2011), with photoperiod duration between 11 and 12 h, density of the flow of photosynthetic photons of 60-70 µmol m^{-2} s^{-1} and temperature of 26 ± 2,0 °C.

Each temporary immersion system consists in two overlapping flasks, one for plant growth and the other as reservoir of the culture medium (below). They are both coupled with a silicon hose, of 6,0 mm diameter and 18,0 cm of length, from the connectors located in the front lower part of each flask. In addition, hydrophobic filters of 0,2 µm were placed to guarantee air sterility within the flasks (figure 1).

The cultivation medium circulated from the lower to the top flask through a hose, due to the pressure of air emitted from a compressor and regulated by a nanometer at 0,1 bar. When the air pressure within the flasks is concluded, the culture medium returns due to gravity to the lower recipient. Between immersions of culture medium, the air is pumped to the recipient that contains the plant tissues to renovate the gaseous atmosphere inside it (Vervit, 2016; Balogun *et al*., 2017), reduce humidity, prevent hyperhydricity and accumulation of toxic gasses (Rocano *et al*., 2017).

Three trials were conducted, with consecutive sequence, which allowed to fix the studied factors in each case and were significant in the trials that are described below:

Figure 1. Temporary immersion systems SETIS™, used in the *in vitro* multiplication of mulberry sprouts, at 28 days of cultivation.

Effect of immersion time. Immersion times of 1,0; 3,0 and 5,0 min. of duration were used, which made up three treatments. From the consulted scientific literature, a frequency of four daily immersions, distributed every six hours (Salas *et al*., 2011), during 28 days, was fixed, under the above-described conditions and culture medium.

Effect of immersion frequency. For the study the best immersion time that resulted from the previous experiment, which was three minutes, was fixed, with different immersion frequencies. Three treatments were made up: 1) three daily immersions every eight hours; 2) control, with four daily immersions every six hours; and 3) six daily immersions every four hours. All of them under conditions and culture media similar to the above-explained experiment.

Effect of cultivation time. The mulberry sprouts were cultivated under the best conditions, determined in the previous experiments. Evaluations were made at 28, 45 and 60 cultivation days. A subculture was made to the fresh culture medium, at 28 days.

Experimental design. A total of three temporary immersion systems SETIS™ per treatment and three repetitions were used, with a complete randomized experimental design. Each system contained 1 000 mL of multiplication culture medium with 30 explants, at a rate of 33,33 mL of culture medium per mulberry explant.

Qualitative variables. After the multiplication period of 28 days, the qualitative characteristics of color and hyperhydricity of the sprouts were evaluated through observation and documented by photographs. The color was determined according to the hexadecimal code of colors (http:// www.cwp.linet.edu/cwis/cwp.html) and hyperhydric sprouts, which showed a turgid and translucent appearance, compared with normal sprouts, were considered.

Quantitative variables. The number of sprouts, number of axillary buds and sprout length (cm), at 28 days of cultivation, was determined. For the sprout length, it was measured from the base to the insertion point of the first leaf, with the aid of a ruler graduated in millimeters.

Statistical processing. The qualitative variables color and hyperhydricity of the sprouts were analyzed through descriptive statistics. In trial one, the effect of immersion time was evaluated, and in two, immersion frequency. The data fulfilled the normality assumptions, according to Bartlett test and variance homogeneity, according to Kolmogorov-Smirnov test. They were statistically processed through a simple classification variance analysis, and mean comparison according to Tukey ($p < 0,05$). Nevertheless, in experiment three, where the effect of the cultivation time was evaluated, the variables did not fulfill the normality assumptions and variance homogeneity, for which the non-parametric Friedman's test was used. The differences among treatments were determined according to Wilcoxon's test ($p < 0,05$). The statistical program SPSS (Statistical Package for the Social Sciences), version PASW Statistics 18 for Windows, was used.

Results and Discussion

Effect of immersion time. The results showed significant differences in the evaluated variables for $p < 0,05$, except in the number of sprouts. It was observed that the number of *M. alba* sprouts, Criolla variety, in the temporary immersion system SETIS™, was

in correspondence with each one of the previously established buds. In addition, the highest number of axillary buds was obtained in treatment 2 (immersion time of three minutes), with value of 4,67. The sprout length in the three treatments exceeded 2,5 cm, but the maximum value was achieved with two minutes of immersion, being higher than 3,0 cm, with significant differences with regards to the others (table 1).

The definition of immersion time is a vital element for each species, if a methodology that implies the use of temporary immersion systems is to be standardized. In this regard, Berthouly and Etienne (2005) stated that the definition of this indicator contributes to the plant tissues achieving maximum nutrient absorption, without reaching their hyperhydration. This is a deleterious physiological disorder, which can lead to an irreversible loss of the *in vitro* regeneration and multiplication capacity, due to the water excess in the intracellular spaces of plant tissues.

The causes that generate hyperhydricity are related to several factors of the *in vitro* environment, such as poor gaseous exchange and high relative humidity in the cultivation flasks, low osmotic potential of the culture media, as well as high concentrations of ions NH_4^+ and Cl^- and of plant regulators, mainly cytokinins (Quiala, 2012).

In the consulted scientific literature it is stated that the immersion should not exceed one or two minutes in woody species, to avoid the presence of sprouts with hyperhydricity symptoms. This time is sufficient to produce increase in the activity of dismutase superoxide and peroxidation of the lipids, which disappear at the end of the immersion phase. Thus, cell death by oxidative stress, induced by the prolonged time of exposure of the explants to the liquid culture medium, is avoided (Salas *et al*., 2011).

Immersion time and frequency are two fundamental factors to achieve the highest number of sprouts and the best plant quality. These indicators are recommended by Castro and González (2002), who when evaluating different immersion frequencies and times in *Eucaliptus grandis* Hill ex Maiden observed that with three minutes of immersion, every 12 h, the highest number of sprouts (11,5) was obtained compared with lower frequencies and one minute of immersion. This result differs from the one obtained in this research, where there was no effect of the immersion time on the number of sprouts.

The number of sprouts formed in this study also contrasts with that reported by Rocano *et al*. (2017) in *Juglans neotrópica* Diels. These authors, using two minutes of immersion every six hours, but in temporary immersion systems BIT®, obtained sprouts of 3,37.

In a work with *Stevia rebaudiana* (Bert.) Bertoni, micropropagated in three immersion systems (BIT®, SETIS™ and RITA®) with two minutes of immersion every 12 h, and a culture medium volume of 10 mL per explant, vigorous plants were achieved with low levels of hyperhydricity, higher average number of leaves and sprouts and, in general, higher multiplication rate than in explants cultivated in semisolid medium (Rosales *et al*., 2018).

It is possible that the shape of the flasks had some influence, when considering that in BIT® a vertical recipient is used, and the space between the explants and the top part of the recipient is higher, compared with the one available in the SETIS, which allows higher availability of gases and space for explant elongation (Rosales *et al.,* 2018).

In this research, 6-BAP and NAA were included in the culture medium, as growth regulators. However, Rosales *et al.* (2018) only added gibberellic acid (AG_3), which induces a large variety of physiological effects; among them, stem elongation, due to the activation of intercalary meristems. In addition, these authors stated that in the initial tests the presence of cytokinins caused negative effects, which seems to indicate that sprouting is due to the temporary immersion system.

Table 1. Effect of immersion time on the multiplication of *M. alba* sprouts, Criolla variety, in temporary immersion system SETIS™.

Treatment	Immersion time, minutes	Number of sprouts	Number of axillary buds	Sprout length, cm
1	1	1,40	3,27[b]	2,59[b]
2	3	1,47	4,67[a]	3,04[a]
3	5	1,43	3,50[b]	2,67[b]
SE ±		0,0592	0,0998*	0,0514*

Different letters in the same column significantly differ according to Tukey's test ($p < 0,05$).

According to Vidal *et al*. (2015) en *Castanea* spp., cultivated in temporary immersion systems RITA®, with three minutes of immersion every four or eight hours, a multiplication coefficient of 1,16 was achieved, which is comparable to the results from this work. In *Eucalyptus camaldulensis* Dehnh, there was the best response, when using 15 min. of immersion every two hours. Under these conditions, no hyperhydricity was observed and a multiplication coefficient 0,74 times higher than that of the system with continuous immersion, was obtained (Mendonça *et al*., 2016).

Regarding this work, there were no hyperhydricity symptoms in the sprouts formed at the different immersion times, with the best response when using three minutes of immersion.

Effect of immersion frequency. With the use of three minutes of immersion and different frequencies (four, six and eight hours), it was observed that the sprouts maintained the typical characteristics of the species, with expanded leaves of green color, smooth surface and undulated edge. Morphophysiological changes, ascribed to the cultivation conditions, such as presence of sprouts with symptoms of hyperhydricity after 28 days of cultivation, were not visible (figure 2).

When utilizing immersion frequencies, every six or eight hours per day, with three minutes of immersion, maximum average values were recorded in the number of axillary buds, with significant differences compared with the treatment with immersion frequency every four hours (figure 3).

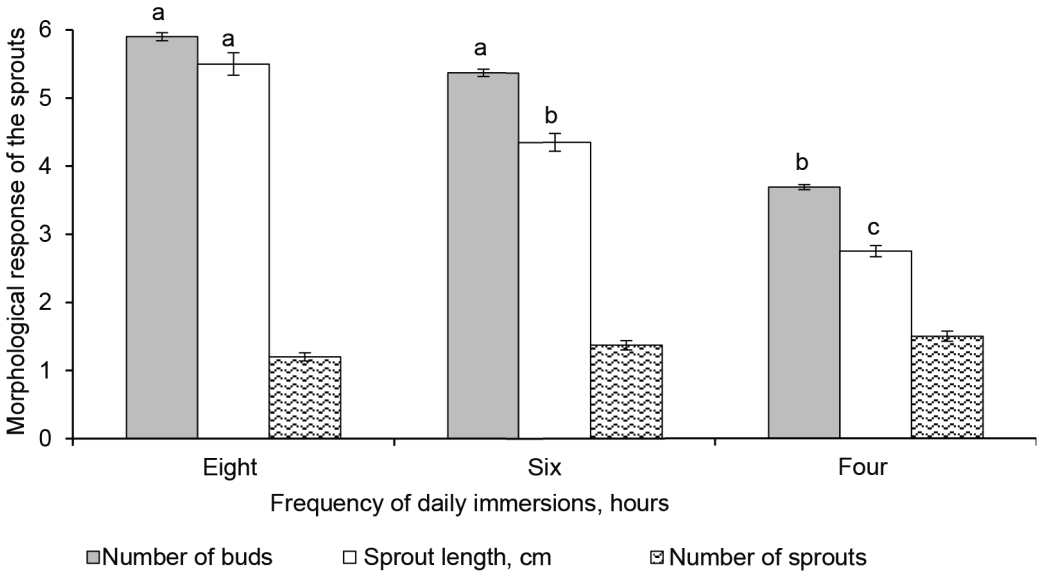
In the variable sprout length, significant differences were shown among the treatments ($P < 0,05$). The highest values were achieved with the lowest number of daily immersions and frequency every eight hours, treatment that differed statistically from the others. On the contrary, in the number of sprouts per explant, there were no significant differences among the treatments (figure 3).

In this experiment the number of buds per sprout (from 3,69 to 5,90) and sprout length (from 2,75 to 5,50 cm), could also be increased, by changing the immersion frequency, from four to every eight hours per day. On the contrary, by decreasing the number of immersions the frequencies or intervals between sprouts are enlarged, and stress is caused in the plant material, which could have stimulated the biological response of the sprouts. During these stress conditions, it is possible that the sprout goes from heterotrophic to mixotrophic nutrition, which stimulates the production of endogenous hormones such as abscisic acid and accelerates the cell division and elongation processes.

González *et al*. (2011) proved that as there is more frequent contact of the explants of *Eucalyptus globulus* Labill. with the culture medium, higher acidification of the liquid culture medium occurs. In low immersion frequencies, the gaseous exchange in photosynthesis and respiration increase, because during immersion the gas diffusion rates in the air are higher than in water.

These results differ from the ones reported by Salas *et al*. (2011), who obtained 11,02 sprouts per

Figure 2. *In vitro* sprouts of mulberry, Criolla variety, obtained with three minutes of immersion and immersion frequencies every eight (A), six (B) and four (C) hours in temporary immersion system SETIS, at 28 days of cultivation.



Bars with different letters in the same variable significantly differ, according to Tukey's test ($p < 0,05$)
*$p < 0,05$

Figure 3. Effect of the immersion frequency on the multiplication of mulberry sprouts, Criolla variety, at 28 days of cultivation.

explant, with approximate length of 11,35 cm in mulberry, Criolla variety, and the utilization of the system of twin flasks, with one minute of immersion and frequency every six hours (four immersions per day). Nevertheless, one of the differences lies on the number of explants. According to these authors, they used ten explants in 500 mL of culture medium, at a rate of 50 mL of culture medium per explant. Meanwhile, in this work the number of explants was triplicated, with 33,33 mL of culture medium as average. When there is a lower number of sprouts, competition decreases, which results in higher availability of light and nutrients. Yet, there is no utilization of the space inside the flask.

This difference in the sprout response, when varying the number of explants per flask, was also proven by Salas *et al.* (2011), who observed that when doubling the number of explants (20), the number of sprouts and their length significantly decreased. Thus, in this work, the low response of tissues could be ascribed to the fact that the number of explants per flask could be triplicated, which could have caused low availability of nutrients and oxygen inside the recipient. Nevertheless, there are factors that could also influence tissue response, such as the type of temporary immersion system used, light intensity inside the growth chamber, explant size, among others.

Businge *et al.* (2017), when using a temporary immersion system BIT® with one minute of immersion every one hour, obtained in the hybrid *Eucalyptus urograndis* (*Eucaliptus grandis* Hill ex Maiden × *E. urophylla* S.T. Blake) a multiplication coefficient 5,75 times higher than in semisolid culture medium, and an average value 1,75 times higher in *Betula pendula* Roth. More recently, Rosales *et al.* (2018) found that in *Stevia rebaudiana* (Bert.) Bertoni, Morita II variety, when using the temporary immersion system BIT® eight sprouts were obtained per plant; while in SETIS®, the mesh on which the explants are placed and its slight inclination to the front, could have decreased the volume of the culture medium in the back part of the flasks and its contact with the explants could have decreased.

This corroborates that the immersion times and frequencies are very variable, because of the large variety of plant species, the micropropagation processes and types of temporary immersion systems used (Gianguzzi *et al*., 2019). Authors like Regueira *et al*. (2018), Carvalho *et al.* (2019) and Vidal and Sánchez (2019), refer the importance of comparing the results in different types of temporary immersion systems and immersion cycles, in order to select for each species the best research equipment and protocol.

It is also possible that the season influenced the sprout response, because the research period coincided with the fructification of this species, which leads to a delay of the physiological processes of the mulber-

ry sprouts. According to Gogoi *et al*. (2017), from November to February *in vitro* flowering in mulberry (*Morus indica* L.) explants occurs, cultivation period that is similar to the one used in this research.

The scientific literature makes reference to different types of explants, such as apices, sprouts, somatic embryos, nodal, mid and basal segments, used in temporary immersion systems. For example, in different clones of *Castanea sativa* Mill, it was reported that the basal explants with calluses produced higher quantity of sprouts, and of higher length than those obtained from nodal and apical segments (Vidal *et al*., 2015).

Palhares *et al*. (2018) proved that the best results in the hybrid *E. urograndis* were achieved when the plants were cultivated in temporary immersion systems, with high light intensity (250 µmol m^{-2} s^{-1}), higher than the one used in this study. According to the authors, this is ascribed to the fact that the plants subject to a high flow of photosynthetic photons reduce, significantly, stomatal conductance, but do not modify transpiration, which results in a higher number of open stomas and the increase of transpiration.

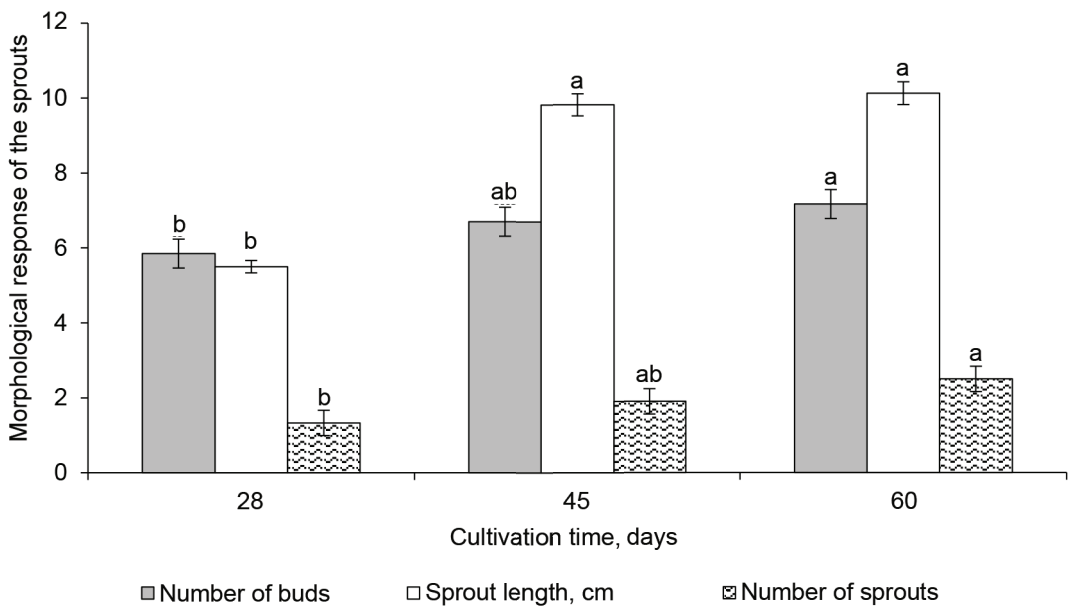
The length of the initial explant is another aspect to be considered, and which could have influenced the length of regenerated sprouts. In this work nodal segments of 1,0 cm of length with an axillary bud were used, lower than the report by Salas *et al*. (2011), who followed the methodology described by Salas *et al*. (2005), and used nodal segments of 2,0 to 3,0 cm of length with one axillary bud.

The integration of these elements should be taken into consideration in future studies, for which upon concluding this experiment it was determined that with the use of three minutes of immersion, with daily frequency every eight hours, higher response is achieved in the analyzed variables.

Effect of cultivation time. When analyzing the effect of cultivation time on the response of the *M. alba* sprouts, Criolla variety, cultivated in the temporary immersion system SETIS™, it was observed that with the increase of cultivation time, the response of the sprouts in all the variables. The minimum values were reached at 28 days of cultivation, with significant statistical differences in sprout length with regards to the other cultivation times ($p < 0,05$). The same did not occur in the evaluations at 45 days of cultivation, in the variables number of sprouts and number of buds.

Although the highest results were obtained at 60 days of cultivation, they did not show significant statistical differences with regards to the average values at 45 days of cultivation in any of the variables (figure 4).

The above-explained facts could have occurred because during the first 28 days of cultivation, plant



Bars with different letters in the same variable significantly differ according to Wilcoxon/Friedman's test ($p < 0,05$)

Figure 4. Effect of cultivation time on the morphological response of mulberry sprouts, Criolla variety, cultivated in temporary immersion system SETIS™.

tissue begins a recovery process, due to the stress that occurs after the establishment of explants in the liquid culture medium. The transference of sprouts to fresh culture medium, at 28 days of cultivation, allowed to maintain the sprouts for a longer time period, with better nutrition. They started to elongate with the subsequent formation of buds and new leaves, which is translated into an increase in the development and multiplication of new sprouts.

In the reviewed literature some authors proved that prolonged cultivation periods affected the number of sprouts. However, Castro and González (2002) obtained the highest coefficient of sprout multiplication in *E. grandis* at six weeks of cultivation, a similar period to the one used in this study.

Upon the conclusion of this experiment, it was determined that cultivation time in the temporary immersion system SETIS™ influenced the multiplication of *in vitro* mulberry sprouts, Criolla variety.

Conclusions

It was proven that the *in vitro* multiplication of mulberry, Criolla variety, in temporary immersion systems SETIS™, is possible. With the use of three minutes of immersion every eight hours, during 45 days of cultivation, the highest response in the number of buds, number of sprouts and sprout length is obtained, without significant statistical differences compared with 60 days of cultivation.

Acknowledgements

The authors thank the financial support of the program of the Secretariat of Higher Education, Science, Technology and Innovation of Belgium (VLIR), coordinated by the University of Gent, in the framework of the project «*In vitro* plant biotechnology for the increase of food security in the eastern region of Cuba», which is developed at the University of Granma.

Authors' contribution

- Jorge Liusvert Pérez-Pérez. Worked on the research conception and design, participated in the data acquisition and interpretation, in the manuscript writing and revision.
- Maylín Fonseca-Yero. Worked on the research conception and design, participated in the data acquisition and interpretation, in the manuscript writing and revision.
- Marisel Bahi-Arevich. Participated in the data acquisition and interpretation and in the manuscript revision.
- Juan José Silva-Pupo. Worked on the research conception, on the acquisition of funding, resources and project management; in addition, participated in the manuscript revision.
- Stefaan Werbrouck. Contributed to the project funding and resource acquisition; in addition, participated in the manuscript revision.

Conflict of interests

The authors declare that there are no conflicts of interests among them.

Bibliographic references

Balogun, M.; Maroya, N.; Taiwo, J.; Chukwunalu, O.; Ajayi, Adeola; Kumar, P. Lava *et al. Clean breeder seed yam tuber production using temporary immersion bioreactors*. Ibadan, Nigeria: IITA, 2017.

Berthouly, M. & Etienne, H. Temporary immersion system: a new concept for use liquid medium in mass propagation. In: A. K. Hvoslef-Eide and W. Preil, eds. *Liquid culture systems for in vitro plant propagation*. Dordrecht, Netherlands: Springer Netherlands. p. 165-195, 2005.

Businge, E.; Trifonova, Adelina; Schneider, Carolin; Rödel, P. & Egertsdotter, Ulrika. Evaluation of a new temporary immersion bioreactor system for micropropagation of cultivars of Eucalyptus, birch and fir. *Forests*. 8 (6):196, 2017. DOI: https://doi.org/10.3390/f8060196.

Carvalho, Lara S. O.; Ozudogru, Aylin; Lambardi, M. & Paiva, L. V. Temporary immersion system for micropropagation of tree species: a bibliographic and systematic review. *Not. Bot. Horti Agrobo.* 47 (2):269-277, 2018. DOI: https://doi.org/10.15835/nbha47111305.

Castro, D. & González, J. Micropropagación de eucalipto (*Eucalyptus grandis* Hill ex Maiden) en el sistema de inmersión temporal. *Agric. Téc.* 62 (1):68-78, 2002. DOI: http://dx.doi.org/10.4067/S0365-28072002000100007.

Georgiev, V.; Schumann, Anika; Pavlov, A. & Bley, T. Temporary immersion systems in plant biotechnology. *Eng. Life Sci*. 14:607-621, 2014.

Gianguzzi, Valeria; Inglese, P.; Barone, E. & Sottile, F. *In vitro* regeneration of *Capparis spinosa* L. by using a temporary immersion system. *Plants, Basel*. 8 (6):177, 2019 DOI: https://doi.org/10.3390/plants8060177.

Gogoi, G.; Borua, P. K. & Al-Khayri, J. M. Improved micropropagation and *in vitro* fruiting of *Morus indica* L. (K-2 cultivar). *J. Genet. Eng. Biotechnol*. 15 (1):249-256, 2017. DOI: https://doi.org/10.1016/j.jgeb.2017.02.005.

González, R.; Ríos, Darcy; Avilés, Fabiola & Sánchez-Olate, M. Multiplicación *in vitro* de *Eucalyptus globulus* mediante sistema de inmersión temporal. *Bosque, Valdivia*. 32 (2):147-154, 2011. DOI: https://dx.doi.org/10.4067/S0717-92002011000200005.

Martín, G. J.; Pentón, Gertrudis; Noda, Yolai; Milera, Milagros; Olivera, Yuseika; Valenciaga, Nuris *et al.* Management of *Morus alba* L. (mulberry). In: Lourdes L. Savón, Odilia Gutiérrez y G. Febles, eds. *Mulberry, moringa and tithonia in animal feed, and other uses. Results in Latin America and the Caribbean*. Mayabeque, Cuba: FAO, ICA. p. 1-25, 2017.

Mendonça, Evânia G.; Santos, B. R.; Stein, Vanessa C.; Beijo, L. A.; Carvalho, H. H. de & Paiva, L. V. The use of continuous, temporary immersion bioreactor system and semisolid culture medium for the production of *Eucalyptus camaldulensis* clones. *Ciência Florestal, Santa Maria*. 26 (4):1211-1224, 2016.

Murashige, T. & Skoog, F. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plant*. 15 (3):473-479, 1962. DOI: https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Palhares, G. A.; Rodríguez-Sánchez, R.; Cid-Ruiz, Mariela; Pina-Trina, D.; Garza-García, Yolanda & González-Olmedo, J. L. Effects of photomixotrophic conditions on plants of *Eucalyptus urograndis* propagated in temporary immersion bioreactors. *Int. J. Agric. Environ. Biotechnol*. 3 (2):558-566, 2018. DOI: http://dx.doi.org/10.22161/ijeab/3.2.33.

Quiala, E. *Efecto de la 6-bencilaminopurina en la morfo-anatomía y la fisiología de brotes de* Tectona grandis *L. cultivados en sistema de inmersión temporal*. Tesis presentada en opción al grado científico de Doctor en Ciencias Agrícolas. Santa Clara, Cuba: Universidad Central "Marta Abreu" de Las Villas, 2012.

Regueira, M.; Rial, E.; Blanco, B.; Bogo, B.; Aldrey, A.; Correa, B. *et al.* Micropropagation of axillary shoots of *Salix viminalis* using a temporary immersion system. *Trees*. 32 (1):61-71, 2018. DOI: https://doi.org/10.1007/s00468-017-1611-x.

Rocano, Mélida N.; Villena, Paulina G. & Peña, Denisse F. Evaluación de los sistemas de cultivo semisólido y BIT en la multiplicación *in vitro* de *Juglans* neotrópica. *Maskana*. 8 (1):103-109, 2017. DOI: https://doi.org/10.18537/mskn.08.01.09.

Rosales, Catalina; Brenes, J.; Salas, Karla; Arce-Solano, Silvia & Abdelnour-Esquivel, Ana. Micropropagación de *Stevia rebaudiana* en sistemas de inmersión temporal para incursionar en la producción hortícola. *Rev. Chapingo Ser. Hortic*. 24 (1):69-84, 2018. DOI: https://doi.org/10.5154/r.rchsh.2017.08.028.

Salas, J. E.; Agramonte, D.; Jiménez-Terry, F.; Pérez, Marta; Collado, R.; Barbón, R. *et al.* Propagación de plantas de *Morus alba* var. Criolla con el uso de sistemas de inmersión temporal. *Biotecnología Vegetal*. 11 (2):77-88, 2011.

Salas-Barbosa, E.; Agramonte, D.; Barbón, R.; Jiménez, F.; Collado, R.; Pérez, Martha *et al.* Propagación *in vitro* de *Morus alba* L. en medio de cultivo semisólido. *Biotecnología Vegetal*. 5 (2):81-87, 2005.

Sarkar, T.; Mogili, T.; Gandhi Doss, S. & Sivaprasad, V. Tissue culture in mulberry (*Morus* spp.) intending genetic improvement, micropropagation and secondary metabolite production. A review on current status and future prospects. In: K. N., ed. *Biotechnological approaches for medicinal and aromatic plants*. Singapore: Springer. p. 467-487, 2018.

Vervit. *SETIS*. Belgium: VERVIT. https://setis-systems.be/about/setis-platform, 2016.

Vidal, Nieves; Correa, B.; Rial, E.; Regueira, M.; Sánchez, Conchi & Cuenca, B. Comparison of temporary and continuous immersion systems for micropropagation of axillary shoots of chestnut and willow. *Acta Hort*. 2015.

Vidal, Nieves & Sánchez, Conchi. Use of bioreactor systems in the propagation of forest trees. *Eng. Life Sci*. 19:896-915, 2019. DOI: https://doi.org/10.1002/elsc.201900041.

Vijayan, K.; Raju, P. J.; Tikader, A. & Saratchnadra, B. Biotechnology of mulberry (*Morus* L.) - An appraisal. *Emir. J. Food Agric*. 26 (6):418-442, 2014. DOI: http://doi.org/10.9755/ejfa.v26i5.15722.

Wani, M. Younus; Mir, M. R.; Baqual, M. F.; Wani, Ab Waheed; Ganie, Nisar A.; Rufaie, S. Zia-ul-Haque. Effect of seed rates on seed germination and seedling growth of mulberry (*Morus* sp.). *Indian J. Exp. Biol*. 57 (12):956-960, 2019.