

Effect of mineral salts and the use of mannitol on the *in vitro* conservation of *Morus alba* L.

Angel Luis Espinosa-Reyes¹ <https://orcid.org/0000-0002-9918-641X>, Juan José Silva-Pupo¹ <https://orcid.org/0000-0003-1049-5740> Jorge Liusbert Pérez-Pérez¹ <https://orcid.org/0000-0003-3372-7559> and Afonso João-Zambela²

¹Centro de Estudios de Biotecnología Vegetal, Facultad de Ciencias Agropecuarias, Universidad de Granma, Apdo. 21. Bayamo. CP 85100. Granma, Cuba. ²Estudiante extranjero, Facultad de Ciencias Agropecuarias, Universidad de Granma, Apdo. 21. Bayamo. CP 85100. Granma, Cuba. E-mail: aespinosar@udg.co.cu, jsilvap@udg.co.cu, jperez@udg.co.cu

Abstract

Objective: To evaluate the effect of mineral salts and the use of mannitol on the *in vitro* conservation of *Morus alba* L., Acorazonada variety.

Materials and Methods: In a first experiment, the treatments included different concentrations of the mineral salts (25, 50, 75 and 100 %) in a basal Murashige and Skoog culture medium, with 0,5 mg mL⁻¹ of 6-benzylaminopurine, naphthaleneacetic acid 0,5 mg L⁻¹ and sucrose 30,0 g L⁻¹. In another experiment, different mannitol concentrations (5,0, 10,0 and 15,0 g L⁻¹) were evaluated in basal MS culture medium. As control, the basal *Murashige* and *Skoog* culture medium was used with 0,5 mg L⁻¹ of 6-benzylaminopurine, naphthaleneacetic acid 0,5 mg L⁻¹ and 30,0 g L⁻¹ of sucrose.

Results: The highest survival values (60,0 and 53,3 %), higher length (3,8 and 3,9 cm) and number of active leaves (1,02 and 1,05) of the preserved material were obtained when using the mineral salts at 75 and 100 %. The utilization of mannitol reduced the growth and number of active leaves, but did not improve the survival of the explants. In both experiments, the explants showed stems without leaves and phenolization symptoms.

Conclusions: The culture medium with the mineral salts at 50 %, 6 benzylaminopurine 0,5 mg L⁻¹, naphthaleneacetic acid 0,5 mg L⁻¹ and 30,0 g L⁻¹ of sucrose favors the *in vitro* conservation of *M. alba*, Acorazonada variety, during six months. The utilization of mannitol reduced growth, but did not improve survival and physical condition of the explants during their *in vitro* conservation.

Keywords: slow growth, germplasm, culture medium, *M. alba*

Introduction

Morus alba is a forage plant, belonging to the family *Moraceae*, introduced in Cuba due to its excellent nutritional qualities for feeding different animal species (Noda-Leyva and Martín-Martín, 2017). It stands out for its high yields. Under different edaphoclimatic conditions it produces between 10 and 12 t DM/ha per year, contains from 20 to 25 % crude protein and the DM digestibility is higher than 80 % (Martín *et al.*, 2014).

It is a crop that contributes to the control of soil erosion. Reports have been written about the *M. alba* potentialities as edible plant and the use of its fresh leaves is recommended (Pentón *et al.*, 2007). Its possibilities to be used in the wine industry and as colorant of food products and preserves, stand out. It is acknowledged for its commercial value in the cosmetic industry. Due to its antioxidant and hypoglycemic phytochemical properties it has been widely used in medicine production (Huh *et al.*, 2017).

The increasing interest in the cultivation of *M. alba* demands the search for alternatives for the conservation of the germplasm of this species in Cuba. It is known that the conservation of plant genetic resources guarantees their possible utilization as potentially useful genetic variation source, and in turn avoids the loss of genetic diversity in agriculture, with the subsequent reduction of the available plant material for the present and future generations. In this regard, Arrigoni-Blank *et al.* (2014) state that the conservation of the diversity contained in a germplasm is the basis of every breeding program, which is reflected on the creation of plants with resistance or tolerance to diverse biotic and abiotic factors, to ensure higher productivity.

The conservation of the *M. alba* germplasm is done in different ways. It can be *in situ*, that is, in the places where it grows naturally, and it can be done *ex situ* through germplasm banks, located in the field, in pots or in greenhouses (Zhang *et al.*, 2019). The *in vitro* methods have also been used

Received: April 22, 2021
Accepted: July 16, 2021

How to cite a paper: Espinosa-Reyes, Angel Luis; Silva-Pupo, Juan José; Pérez-Pérez, Jorge Liusbert & Zambela, Afonso João. Effect of mineral salts and the use of mannitol on the *in vitro* conservation of *Morus alba* L. *Pastos y Forrajes*. 44:e120, 2021.

This is an open access article distributed in Attribution NonCommercial 4.0 International (CC BY-NC4.0) <https://creativecommons.org/licenses/by-nc/4.0/>
The use, distribution or reproduction is allowed citing the original source and authors.

(Arias-Padró *et al.*, 2012; Vijayan and Sangannavar, 2020).

In vitro conservation has been profiled as a valuable alternative for the conservation of plant germplasm. This form of preserving the plant material relieves plants from the risks that occur in the field, reduces costs, ensures the maintenance of genetic fidelity and facilitates the exchange of germplasm (Arrigoni-Blank *et al.*, 2014).

There are two main forms to carry out *in vitro* conservation: conservation by minimum growth and cryopreservation. The objective of the former is to decrease *in vitro* plant growth through the decrease of metabolism and cell division, for which the optimum cultivation conditions and the composition of the culture medium are disturbed (González-Arno *et al.*, 2017). Among the disturbances in the composition of culture media, the reduction of mineral nutrients and the addition of growth regulators and osmotic regulators are more frequent (Jiménez-Mariña *et al.*, 2016; Kovalchuk *et al.*, 2018).

The available *M. alba* germplasm in Cuba comprises, approximately, 22 varieties, which are preserved at the Pastures and Forages Research Station Indio Hatuey, introduced from Costa Rica, Ethiopia, Brazil, South Korea, China and Spain (Reino-Molina *et al.*, 2017). It is preserved in germplasm banks in the field, with loss risk due to natural disasters and the attack by pests and diseases, situation that demands the search for alternatives to guarantee its conservation.

The bibliographic review showed that the most widely used method for conservation by minimum growth of the genus *Morus* is temperature decrease. No results were found related to the use of osmotic regulators, reduction of nutrient content or other changes in the composition of the culture medium or in the ambient conservation conditions. That is why to justify the results obtained in this work studies conducted in other crops, and which are related to the use of mannitol and management of mineral salts for *in vitro* conservation, were utilized.

This study was conducted in order to evaluate the effect of mineral salts and the use of mannitol on the *in vitro* conservation of *M. alba*, Acorazonada variety.

Materials and Methods

Location. The research was conducted in the Plant Biotechnology Research Center of the University of Granma, in the period comprised between January,

2020, and September, 2020. The Acorazonada variety, from the germplasm bank of the Plant Biotechnology Research Center of the University of Granma, was used.

Plant material and culture media. For the *in vitro* establishment, nodal segments of 1,5-2,0 cm of length, which come from sprouts obtained from stakes sprouted under semicontrolled conditions, were used as explants. They were superficially disinfected with sodium hypochlorite at 1 % of active chlorine during 20 min and were later rinsed four times with sterile distilled water under aseptic conditions. In all the experiments, as basal culture medium (BM) the MS salts and minerals (Murashige and Skoog, 1962), 6-BAP (6-benzylaminopurine) 0,5 mg L⁻¹, NAA (naphthaleneacetic acid) 0,5 mg L⁻¹ and 30,0 g L⁻¹ of sucrose, solidified with 6,0 g L⁻¹ of agar E (Biocen) and pH 5,7 (Salas *et al.*, 2011), were used. The explants were incubated at 25 ± 2 ° C, with a regime of 16 light hours and photosynthetic photon flux density (PPFD) of 62-68 μmol m⁻²s⁻¹ and eight hours of darkness, during 30 days.

The plant material multiplication was carried out on a culture medium similar to the one described for the *in vitro* establishment stage. Three subcultures were done until having the necessary plant material quantity to conduct the conservation experiments that are described below.

Experiment 1. Effect of mineral salts on the in vitro cultivation of M. alba. The content of mineral salts (macro salts) of the MS culture medium (Murashige and Skoog, 1962) was distributed and the following treatments were made up: T1) MS salts at 25 %, T2) MS salts at 50 %, T3) MS salts at 75 % and T4) MS salts at 100 %. T4, which contained the basal culture medium with the salts at 100 % of their concentration, was established as control.

Experiment 2. Effect of mannitol on the in vitro conservation of M. alba. Different concentrations of mannitol in the culture medium were evaluated and the following treatments were made up: T1) basal medium, T2) basal medium + mannitol (5,0 g L⁻¹), T3) basal medium + mannitol (10,0 g L⁻¹) and T4) basal medium + mannitol (15,0 g L⁻¹). T1, which contained the basal culture medium without mannitol, was established as control.

Experimental design. In the two experiments a complete randomized design was applied and five tubes were used per treatment, for a total of 30 tubes per essay (150 x 25 mm), with 10 mL of culture medium and an explant per tube.

Measurements. At six months the survival of the explants was evaluated (percentage calculation from the number of live explants) by visual observation. The length of the sprouts (cm) was measured through a graduated ruler and the number of active leaves (green leaves) was quantified.

Statistical analysis. The data normality was tested by the Kolmogorov-Smirnov test and variance homogeneity, through Levene's test. As there was normality and variance homogeneity, simple variance analysis was done. In the cases in which there were significant differences among the means, Tukey's multiple range comparison test ($p = 0,05$) was applied. The variables, expressed in percentage, were processed through a proportion difference analysis. The statistical package Infostat 2017 (Di Rienzo *et al.*, 2017) was used.

Results and Discussion

During the first three months of conservation, the explants showed in all the treatments very good physical conditions: leaves of green color, fully extended, practically all in active status and without symptoms of hyperhydricity. Nevertheless, once six months passed, the physical conditions of the explants were much deteriorated in all the treatments. Survival was affected in all of them (table 1), with significantly lower values when using the salts at 25 %. The treatments in which the mineral salts were used at 50 % of their concentration or more, did not show significant differences among them.

Sprout length was significantly higher in the treatments with higher concentration of mineral salts (75 and 100 %). In the treatment with the salts at 25 %, the sprout length was significantly lower than the other treatments. The number of active leaves at six months of cultivation drastically decreased in all the treatments, and reached zero in the ones that

had lower salt concentrations (25 and 50 %). The sprouts showed green color, but practically without leaves, result that allows to assume that the nutrient concentration is very low to supply the sprouts.

The results of this experiments show a marked influence of the concentration of inorganic salts of the medium on the survival and development of the *in vitro* *M. alba* plants, Acorazonada variety. There was a general trend to decrease the value of the morphological variables, as the concentration of inorganic salts decreased. In this regard, Kovalchuk *et al.* (2018) acknowledge that mineral nutrients are among the main components of the culture media used in the cultivation of plant tissues.

Martínez-Villegas *et al.* (2015) state that the effect of the concentration of inorganic salts on the explant growth is closely related to the osmotic potential of the culture medium. They indicate that as such potential is reduced water and nutrient absorption is lower, which hinders sprout growth and multiplication. Nevertheless, it is considered that in this research the concentration of mineral salts used do not generate a hydric potential that affects water and nutrient absorption, and ascribe the differences in explant growth to the nutrient availability in the evaluated culture media.

These results coincide with those reported by Castilla-Valdés *et al.* (2020), who obtained at six months of *in vitro* conservation of *Coffea arabica* L significantly lower values, regarding survival and number of leaf pairs in the culture media. The above-cited authors used the nutrient salts at 25 and 50 %, unlike other treatments with higher concentration of salts. Also in agreement with the results of this study are the works by Jiménez-Mariña *et al.* (2016) during the *in vitro* conservation of carnation (*Dianthus caryophyllus* L.), obtaining significant differences in the survival of plants cultivated in culture medium with the MS salts,

Table 1. Effect of the concentration of mineral salts in the culture medium on the *in vitro* conservation of *M. alba*, Acorazonada variety.

MS salts, %	Survival, %	Sprout length, cm	Number of active leaves
25	40,0 ^b	1,1 ^c	0 ^c
50	56,6 ^{ab}	2,6 ^b	0 ^c
75	60,0 ^a	3,8 ^a	1,0 ^b
100	53,3 ^{ab}	3,9 ^a	1,1 ^b
SE ±		0,060	0,130
P - value		p < 0,001	p < 0,0001

Means with different letters per column significantly differ, according to Tukey's test ($p < 0,05$)
SE: standard error

reduced to 25 % of their concentration compared with the other treatments, after three months of cultivation.

Effect of mannitol on the *in vitro* conservation of *M. alba*. The inclusion of mannitol in the culture medium did not influence explant survival. Table 2 shows that there were no statistical differences with regards to the control group or among the different evaluated mannitol concentrations.

The mannitol concentrations used significantly reduced sprout length and number of active leaves, with significant differences among them and with regards to the control treatment. This could be due to the effect caused by mannitol on the decrease of the osmotic and hydric potential of the culture medium, which hinders water and nutrient absorption and causes reduction in plant growth. Regarding the above-explained facts, García-Águila *et al.* (2007) state that mannitol is an osmotic agent that exerts its limiting effect on explant growth because of reduction of water and nutrient absorption from the culture medium.

The *in vitro* plants at the moment of evaluation showed green stems, practically without active leaves and with losses of the apical bud in some cases. The culture media in all the treatments showed phenolization symptoms.

The results partially coincide with reports by other authors who have referred the use of mannitol for *in vitro* plant conservation. Rayas-Cabrera *et al.* (2019) found that the use of mannitol at concentrations of 1,0, 1,5 and 2,0 % in the *in vitro* conservation of two varieties of *Ipomoea batatas* (L.) Lam., reduced growth, without affecting the survival of the plants preserved during eight months. Alvim *et al.* (2020) reported the reduction of growth of the aerial part of the plant, when preserving *in vitro* *Amburana cearensis* (Allemão) A.C.Smith. (*Fabaceae*), native

species of Brazil, and the decrease of survival and number of active leaves since 120 days of cultivation in the media with higher mannitol concentrations (1,6 and 2,4 %).

Rayas-Cabrera *et al.* (2020) when preserving *in vitro* cultivars of *Dioscorea alata* L. determined that the use of mannitol at 1,5 % in the culture medium favored survival and number of sprouts per explants. These authors acknowledge that its use increased survival of the preserved material during the recovery process, effect that is also favored by the presence of activated carbon in the culture medium of conservation.

The results obtained in this research indicate that the inclusion of mannitol in the culture medium at the evaluated concentrations, in spite of reducing growth, did not improve survival and physical condition of the explants. This suggests the need to evaluate the effectiveness of this osmotic regulator with new concentrations, its combination with other substances and environmental conditions.

Conclusions

The culture medium with mineral salts at 50 %, 6-benzylaminopurine 0,5 mg L⁻¹, naphthaleneacetic acid 0,5 mg L⁻¹ and 30,0 g L⁻¹ of sucrose favors the *in vitro* conservation of *M. alba*, Acorazonada variety, during six months. The use of mannitol reduced growth, but did not improve survival and physical condition of the explants during their *in vitro* conservation.

Acknowledgements

The authors thank the Program VLIR-USO, of Belgium, which contributed the funds for the execution of the Project «*In vitro* plant biotechnology for the increase of food security in the eastern region of Cuba».

Table 2. Effect of the use of mannitol on the *in vitro* conservation of *M. alba*, Acorazonada variety.

Mannitol, g L ⁻¹	Survival, %	Sprout length, cm	Number of active leaves
0	60,0	3,9 ^a	1,1 ^a
5,0	50,0	1,8 ^b	1,2 ^a
10,0	60,0	0,7 ^c	0,4 ^b
15,0	63,3	0,4 ^c	0,2 ^b
SE ±	ns	0,090	0,130
P - value		p < 0,0001	p < 0,0001

Means with different letters per column significantly differ, according to Tukey's test for p < 0,05
ns: There are no significant differences.

Conflict of interests

The authors declare that there is no conflict of interests among them.

Contribución de los autores

- Angel Luis Espinosa-Reyes. Participated in the elaboration of the research project, setting up and evaluation of the experiments, data processing and paper writing.
- Jorge Liusbert Pérez-Pérez. Participated in the elaboration of the research project, data processing and paper writing.
- Juan José Silva-Pupo. Participated in the elaboration of the research project, experiment evaluation and data statistical analysis and paper writing.
- Afonso João-Zambela. Participated in the search for and preparation of the plant material, setting up and evaluation of the experiments, data taking and search for bibliographic information.

Bibliographic references

- Alvim, B. F. M.; Souza, Ana V. V. de; Lima-Brito, A.; Fonseca, Priscila T.; Soares, Taliane L. & Santana, J. R. F. de. Conservação *in vitro* de *Amburana cearensis* (Fabaceae). *Cienc. Rural*. 50 (7), 2020. DOI: <https://doi.org/10.1590/0103-8478cr20190729>.
- Arias-Padró, María D.; Frattarelli, Andrea; Sgueglia, Alessandra; Condello, E.; Damiano, Carmine & Caboni, Emilia. Cryopreservation of white mulberry (*Morus alba* L.) by encapsulation-dehydration and vitrification. *Plant Cell Tiss. Organ. Cult.* 108:167-172, 2012. DOI: <https://doi.org/10.1007/s11240-011-0017-5>.
- Arrigoni-Blank, María de F.; Tavares, Fernanda F.; Blank, A. F.; Santos, Maria C.; Menezes, Thays S. A. & Santana, Aléa D. D. de. *In vitro* conservation of sweet potato genotypes. *Scientific World J.*, 2014. DOI: <https://doi.org/10.1155/2014/208506>.
- Castilla-Valdés, Yanelis; González-Vega, María E. & Espinosa-Torres, Llayma. *In vitro* conservation of coffee-tree (*Coffea arabica* L.) by decreasing mineral salts in the culture medium. *Cult. Trop.* 41 (1):e04. http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S0258-59362020000100004&lng=es&nrm=iso, 2020.
- Di Rienzo, J. A.; Casanoves, F.; Balzarini, Mónica G.; González, Laura A.; Tablada, M. & Robledo, C. W. *InfoStat versión 2017*. Córdoba, Argentina: Grupo InfoStat, FCA, Universidad Nacional de Córdoba. <http://www.infostat.com.ar>, 2017.
- García-Águila, Leanis; Fera, M. de & Acosta, Karen. Aspectos básicos de la conservación *in vitro* de germoplasma vegetal. *Biot. Veg.* 7 (2):67-79. <https://revista.ibp.co.cu/index.php/BV/article/view/359>, 2007.
- González-Arno, María T.; Dolce, Natalia; González-Benito, María E.; Castillo-Martínez, C. R. & Cruz-Cruz, C. A. Approaches for *in vitro* conservation of woody plants germplasm. In: *Biodiversity and conservation of woody plants*. Sustainable Development and Biodiversity. Vol. 17. New York: Springer. p. 355-419, 2017.
- Huh, Y. S.; Lee, J. K. & Nam, S. Y. Improvement of *ex vitro* acclimatization of mulberry plantlets by supplement of abscisic acid to the last subculture medium. *J. Plant Biotechnol.* 44 (4):431-437, 2017. DOI: <http://dx.doi.org/10.5010/JPB.2017.44.4.431>.
- Jiménez-Mariña, Liudmila; Silva-Pupo, J. J.; Borges-García, M. & Fonseca-Arias, Milvia. Conservación *in vitro* del cultivo de clavel español (*Dianthus caryophyllus* L.) a partir de sales minerales. *Agron. Mesoam.* 27 (1):177-181, 2016. DOI: <http://dx.doi.org/10.15517/am.v27i1.21897>.
- Kovalchuk, Irina Y.; Mukhitdinova, Zinat; Turdiyev, T.; Madiyeva, Gulnara; Akin, M.; Eyduran, E. *et al.* Nitrogen ions and nitrogen ion proportions impact the growth of apricot (*Prunus armeniaca*) shoot cultures. *Plant Cell Tiss. Organ. Cult.* 133:263-273, 2018. DOI: <https://doi.org/10.1007/s11240-018-1379-8>.
- Martín, G. J.; Pentón, Gertrudis; Noda, Yolai; Contino, Y.; Díaz, Maykelis; Ojeda, F. *et al.* Comportamiento de la morera (*Morus alba* L.) y su impacto en la producción animal y la crianza de gusanos de seda en Cuba. *Rev. cubana Cienc. agric.* 48 (1):73-78. <http://www.redalyc.org/articulo.oa?id=193030122016>, 2014.
- Martínez-Villegas, Ylvi M.; Andrade-Rodríguez, María; Colinas-León, M. Teresa; Villegas-Torres, Ó. G.; Castillo-Gutiérrez, A. & Alia-Tejacal, I. Efecto de las sales inorgánicas del medio de cultivo en el crecimiento de pascuíta (*Euphorbia leucocephala* L.) *Rev. fitotec. mex.* 38 (4):369-374. http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S0187-73802015000400004&lng=es&nrm=iso, 2015.
- Murashige, T. & Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum.* 15 (3):473-497, 1962. DOI: <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>.
- Noda-Leyva, Yolai & Martín-Martín, G. J. Efecto de la distancia de siembra en el rendimiento de *Morus alba* (L.) var. yu-12. *Pastos y Forrajes.* 40 (1):23-28. http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S0864-03942017000100003&lng=es&nrm=iso, 2017.

- Pentón, Gertrudis; Martín, G. J.; Pérez, A & Noda, Yolaí. Comportamiento morfoagronómico de variedades de morera (*Morus alba* L.) durante el establecimiento. *Pastos y Forrajes*. 30 (3):315-325. http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S0864-03942007000300003&lng=es&nrm=iso, 2007.
- Rayas-Cabrera, Aymé; López-Torres, J.; Medero-Vega, V. R.; Basail-Pérez, Milagros; Santos-Pino, Arletys & Martínez-Pérez, Marilín. Conservación *in vitro* de cultivares de ñame (*Dioscorea alata* L.) bajo condiciones de crecimiento mínimo. *Rev. Agricultura Tropical*. 6 (1):33-40. http://ojs.inivit.cu/index.php?journal=inivit&page=article&op=view&path%5B%5D=133&path%5B%5D=AT06012020_5BV21, 2020.
- Rayas-Cabrera, Aymé; López-Torres, J.; Medero-Vega, V. R.; Basail-Pérez, Milagros; Santos-Pino, Arletys & Gutiérrez-Sánchez, Yenisey. Conservación *in vitro* de cultivares de *Ipomoea batatas* (L.) Lam por crecimiento mínimo con el uso de manitol. *Biot. Veg.* 19 (1):43-51. http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S2074-86472019000100043&lng=es&nrm=iso, 2019.
- Reino-Molina, J. J.; Montejo-Valdés, Laura A; Sánchez-Rendón, J. A. & Martín Martín, G. J. Características seminales de cinco variedades de morera (*Morus alba* L.) cosechadas en Matanzas, Cuba. *Pastos y Forrajes*. 40 (4):276-280. http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S0864-03942017000400003&lng=es&nrm=iso, 2017.
- Salas, J. E.; Agramonte, D.; Jiménez-Terry, F.; Pérez, M.; Collado, R.; Barbón, R. *et al.* Propagación de plantas de *Morus alba* var. Criolla con el uso de sistemas de inmersión temporal. *Biot. Veg.* 11 (2):77-88. <https://revista.ibp.co.cu/index.php/BV/article/view/220/196>, 2011.
- Vijayan, K. & Sangannavar, P. A. Role of biotechnology in genetic conservation of mulberry plants for crop improvement. *Gene Technol.* 10 (S3):7530. <https://www.longdom.org/open-access/role-of-biotechnology-in-genetic-conservation-of-mulberry-plants-for-crop-improvement.pdf>, 2020.
- Zhang, Y.; Zuo, D.; Zhang, J.; Wang, Z. & Chen, C. Development of a mulberry core collection originated in China to enhance germplasm conservation. *Crop Breed. Appl. Biotechnol.* 19 (1):55-61, 2019. DOI: <https://doi.org/10.1590/1984-70332019v19n1a08>.