Scientific Paper

Influence of in vitro plant size and substrate type on the acclimatization of *Morus alba* L.

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Abstract

The objective of this study was to evaluate the influence of size and substrate type on the acclimatization of *in vitro Morus alba* L. plants. For such purpose, three trials were conducted: in the first one *in vitro* plants were evaluated grouped according to their sizes, which constituted the treatments (T1: 1,5-2,5 cm; T2: 2,6-3,5 cm and T3: higher than 3,5 cm); in the second one, plants with a length between 2,5 and 3,0 cm were selected and three formulations of substrate mixtures were evaluated: T1: soil (70 %)-cattle manure (20 %)-zeolite (10 %), T2: soil (45 %)-cattle manure (45 %)-zeolite (10 %), T3: soil (90 %)-zeolite (10 %); and in the third trial the substrate type was evaluated on the *in vitro* plant growth under nursery conditions, with the same treatments. A simple variance analysis was carried out, and Tukey's multiple range comparison test was applied for $p \leq 0,05$. The statistical package Infostat (2017) on Windows® was used. The increases in sprout length and survival (93,6 %) were significantly higher when *in vitro* plants from 1,5 to 2,5 cm long were used. Using the substrate mixtures T1 and T2 a survival higher than 80 % was obtained; just like higher length, number of leaves and leaf size in the *in vitro* plants in the initial stage of acclimatization and under nursery conditions. It is concluded that with the use of *in vitro* plants of size between 1,5 and 2,5 cm and the substrates of treatments T1 and T2 during acclimatization, high survival and higher growth and development of the *M. alba* plants were achieved.

Keywords: growth, tissue culture, survival, *in vitro* plants

Introduction

Mulberry (*Morus alba* L.) was introduced in Cuba with forage purposes for animal feeding, and it has been proven to have excellent nutritional qualities for feeding different animal species (Noda-Leyva y Martín-Martín, 2017). This plant has high adaptive capacity to different edaphoclimatic conditions, can produce between 10 and 12 t of dry matter per hectare per year, contains from 20 to 25 % crude protein, and dry matter digestibility is higher than 80 % (Martín *et al*., 2014). In addition, it is renowned for its commercial value in the cosmetic and medicinal industry, and its physical-chemical antioxidant and hypoglycemic properties have been widely used in drug production (Huh *et al*., 2017).

Mulberry propagation is generally done by stakes; however, depending on the cultivar there are certain aspects –such as the low survival and multiplication rate, as well as the rooting difficulty– which limit the propagation of this plant species with productive purposes (Castro-Ramírez, 2010).

The *in vitro* propagation of plant species has emerged as a valuable alternative for the propagation of species of economic and ornamental interest, because it allows the production of large quantities of plants in a relatively short time period; it is an excellent tool for the preservation and recovery of species that have significantly decreased their populations and for breeding.

Mulberry breeding programs are aimed at the increase of foliage yields. Nevertheless, due to the high heterogeneity and long periods for plant regeneration, conventional breeding techniques are limited; for such reason it has been necessary to complement them with modern biotechnological techniques, such as tissue culture, molecular DNA recombination techniques and molecular markers (Vijavan *et al*., 2014).

The success of tissue culture techniques depends essentially on having a well-established protocol that includes the different stages of the process, such as plant propagation, rooting and acclimatization (Resende *et al*., 2015).

In Cuba diverse research works have been conducted aimed at establishing an efficient protocol of *in vitro* mulberry propagation, among which the ones conducted by Salas *et al*. (2005) and Salas

et al. (2011), whose objective is the propagation of this plant through organogenesis in semisolid culture medium and in temporary immersion systems. Likewise, it should be stated that although the studies related to acclimatization of mulberry plants from tissue culture are scarce, Salas *et al*. (2005) obtained survival values between 60 and 70 % when they evaluated the substrate effect, besides the morphological traits of the plants under field conditions.

The plants that come from tissue culture, generally, require treatments to prevent their death before transferring them to the *ex vitro* conditions, because those produced *in vitro* are incapable of standing the environmental changes which they face, because they have been developed in an aseptic environment, with minimum temperature variations, high humidity and nutrient availability and low carbon dioxide concentration. For such reasons the transference must be gradually done; during this stage a progressive return to the autotrophic functioning of *in vitro* plants occurs, as well as the recovery of normal morphological and physiological characteristics (Silva *et al*., 2017).

Taking all the above-mentioned facts into consideration, the objective of this study was to evaluate the influence of size and substrate type on the acclimatization of *in vitro M. alba* plants.

Materials and Methods

The study was conducted in the acclimatization area of the Plant Biotechnology Study Center belonging to the School of Agricultural Sciences, in the University of Granma (UDG) –Cuba–, in the period from October, 2017, to June, 2018.

Three trials were conducted; the plant material used in the first and second ones consisted in *in vitro M. alba* plants of the acorazonada variety, rooted during 60 days in a culture medium constituted by the Murashige and Skoog (1962) salts, supplemented with indole butyric acid 1 $mg.L^{-1}$, thiamine 1 $mg.L^{-1}$, myo-inositol 100 $mg.L^{-1}$, sucrose 20 g. L^{-1}, and solidified with agar 6 $g.L^{-1}$. The pH was adjusted to 5,7. In the third experiment mulberry plants acclimatized during 30 days under *ex vitro* conditions, with a length of 7,03 cm and six leaves, as average were used.

All the experiments were conducted in the adaptation house of the Plant Biotechnology Study Center of the School of Agricultural Sciences, under a black saran shade cloth, to regulate illumination.

In the first and second experiment the plants were extracted from the culture flasks. The roots were carefully washed with abundant water, to eliminate the culture medium remains; and were placed in a tap water container, to prevent their dehydration, during a period from 13 to 14 hours before being transplanted to the *ex vitro* conditions.

At the moment of planting the root system of the *in vitro* plants was completely covered with the substrate, slightly pressing to guarantee that they became fixed. Polyurethane trays of 70 cells were used, with a capacity in each cell of 120 cm^3 of substrate; a vitroplant was sown in each cell, and all of them were covered with transparent glass flasks during seven days, in order to maintain a high relative humidity and thus decrease water loss. After that time the cover was removed.

In the third experiment black polyethylene bags were used, filled up to three quarters with the substrate mixture to be evaluated. Irrigation in all the trials was daily performed with a watering pot, until the substrate was saturated.

For all the trials a completely randomized design was used with three replicas. In each repetition 15 vitroplants were sown, for a total of 45 per treatment.

In trial 1 the influence of size on the acclimatization of *in vitro* mulberry plants was studied. They were classified according to their length into three groups and this allowed to make up the following treatments: T1: small (1,5-2,5 cm), T2: medium (2,5- 3,5 cm), T3: large (> 3,5 cm). A mixture of soil (70 %)-cattle manure (20 %)-zeolite (10 %) was used as substrate.

On the other hand, in trial 2 the influence of the substrate type on the acclimatization of the *in vitro* mulberry plants was studied. The plants were selected with the highest possible homogeneity with regards to their length (2,5-3,0 cm). Three substrate mixtures were evaluated, selected according to the results obtained in other studies with woody species (Cholo-Masapanta y Delgado-Rodríguez, 2011). The treatments were the following: T1: soil (70 %)-cattle manure (20 %)-zeolite (10 %), T2: soil (45 %)-cattle manure (45 %)-zeolite (10 %), T3: soil (90 %)-zeolite (10 %).

Meanwhile, in trial 3, the effect of substrate type on the growth of the plants from the acclimatization stage under nursery conditions was studied. The mulberry plants acclimatized during 30 days were carefully extracted from the trays to preserve the substrate (mound), and they were individually

placed in black polyethylene bags which contained different substrate mixtures. The following treatments were evaluated: T1: soil (70 %)-cattle manure (20 %)-zeolite (10 %), T2: soil (45 %)-cattle manure (45 %)-zeolite (10 %), T3: soil (90 %)-zeolite (10 %). Thirty days after sowing the 45 plants from each plant were taken, and the following variables were evaluated:

- Survival (%): for its evaluation the total sown plants were considered. The following formula was used:

$$\text{Survival} = \frac{\text{NPV}}{\text{NTP}} \text{ X } 100$$

  NPV: number of live *in vitro* plants
  NTP: total number of sown *in vitro* plants
- Plant length (cm): it was measured with a ruler, from the stem base to the apex of the top leaf.
- Number of leaves per plant: it was determined by counting the number of fully extended leaves.
- Leaf length (cm): it was measured with a ruler, from the insertion of the limbo with the petiole to the leaf apex.
- Leaf width (cm): it was measured with a ruler, on the broader part of the leaf.

Statistical analysis. The normality was proven by the Kolmogorov-Smirnov test, and the variance homogeneity, through Levene's test. For the variables that fulfilled the assumptions a variance analysis was performed; while the variable survival was processed through a proportion difference analysis. In the cases where there were significant differences among the means Tukey's multiple range comparison test was applied for $p \leq 0,05$. The statistical package Infostat 2017 (Di Rienzo *et al*., 2017) was used.

Results and Discussion

Influence of size on the acclimatization of *in vitro* mulberry plants

The adequate size and good quality of *in vitro* plants is an important factor to be considered during the acclimatization stage, because survival, growth rate and final production in the field stage depend on that.

The *in vitro* plants cultivated during 30 days under *ex situ* conditions showed differences in survival; significantly higher values were reached with those of lower length (1,5-2,5 cm) with regards to the other treatments, which did not differ between them (table 1). In all the treatments survival was considered high; and the *in vitro* plants showed turgid, fully extended leaves, with the dark green color characteristic of the species.

The good results in this indicator could be ascribed to the adequate management during this stage, to the environmental conditions under which the trial was conducted and to the characteristics of the species, which make it resistant to the *ex vitro* conditions. This coincides with the results obtained by Pérez-Alonso *et al*. (2016) in the acclimatization of *in vitro Aloe vera* L. plants and by Salas *et al*. (2011) in mulberry vitroplants, who consider that the culture conditions under which the acclimatization process was developed are determinant to obtain high survival values.

The other morphological variables did not show significant differences in the treatments at the end of the period; however, it must be emphasized that the smaller size plants reached the highest increases in length (5,38 cm) and in number of leaves, which indicates that they were capable of recovering faster from the initial stress originated by the transplant to *ex vitro* conditions and restart growth and development in less time, compared with those of higher size; this could be associated with a better makeup and quality of the foliage and of the root system of lower-size plants.

Palhares (2004), when evaluating the influence of *in vitro* plant size during the acclimatization of *Eucalyptus urograndis* obtained in temporary immersion systems, achieved the highest survival percentage (63 %) and with the highest values in the growth variables (length, number of leaves

Table 1. Influence of size on the acclimatization of *in vitro* mulberry plants 30 days after sowing.

Treatment	Survival, %	Length, cm	Leaves/plant	Leaf length, cm	Leaf width, cm
Small	93,6[a]	7,68	6,65	3,77	2,83
Medium	85,4[b]	7,82	6,13	3,58	2,66
Large	84,8[b]	8,05	6,75	3,76	2,74
SE ±	2,06	0,08	0,09	0,10	0,06

Means with different letters per column significantly differ, according to Tukey's test, for $p < 0,05$.

and number of emitted roots) in the seedlings with lower sizes. The authors concluded that, seemingly, size is not the variable with higher effect on the survival of the *E. urograndis* seedlings during the acclimatization, but the quality of the different organs that compose it.

The leaf quality and total number of the plants have a direct effect on the success of acclimatization. In all the treatments an increase was observed in the emission of new leaves, without significant differences among them, and there were no differences either in leaf width and length. Most studies of the leaf tissue of the plants that come from *in vitro* conditions refer that the mesophyll of these leaves has little-developed palisade tissue, generally formed by a single cell layer and fundamentally composed by spongy tissue with large intercellular spaces (Molina-El-Hage *et al*., 2008; Soares and Savonitti, 2016). This limits them from having optimum functionality, for which the emission of new leaves under *ex vitro* conditions directly causes higher seedling growth and development, because they reach higher photoautotroph activity.

On the other hand, Albany *et al*. (2006), when establishing a methodology of *in vitro* propagation of *Aloe vera*, evaluated the influence of the vitroplant size (small: < 5 cm, medium: 5-10 cm, large >10 cm) during the acclimatization stage. There were no significant differences in the survival percentage, with regards to the size of the *in vitro* plants. After 60 days of adaptation to the *ex vitro* conditions all the plants were alive and showed uniform growth, in spite of the size differences at the moment of transplant.

Influence of substrate type on the acclimatization of mulberry vitroplants

Among the factors with higher influence on the acclimatization of the *in vitro* plants is the substrate type and composition, which determines an adequate humidity retention and the chemical components to provide the plant with water and nutrients; and, at the same time, exerts a significant influence on the architecture of the root system, which influence the nutritional status and water translocation in plants. That is why it is necessary to pay attention to their selection and use.

The survival of vitroplants did not show significant differences among the treatments, with values higher than 80 %, which can be considered acceptable for the conditions under which the trial was conducted (table 2).

These results could be associated to the management and conditions under which the plants were maintained during the acclimatization: adequate humidity, which was guaranteed by the irrigation frequency and type and by the use of covers in the cultivation house; moderate illumination, through the shade mesh; and maintenance of the *in vitro* plants during the multiplication and rooting stage in growth chamber with sunlight, with a photoperiod of 13-11 h of light/darkness.

Manure, one of the components used in the substrate mixtures, when being added to the soil, contributes to improving its biological and physical-chemical properties, because it is an important source of energy and nutrients for the edaphic ecosystem (Cairo-Cairo and Álvarez-Hernández, 2017).

The addition of zeolite to substrates favors aeration, nutrient absorption by the plant and better water supply, which leads to obtaining a plant of excellent physiological quality in the acclimatization stage (Urbina-Sánchez *et al*., 2006).

The substrate type used had significant effect on *in vitro* plant growth and development in the cultivation house. The best results for all the variables were obtained by using the substrate composed by 70 % soil-20 % cattle manure and 10 % zeolite (T1), without significant differences from T2 (mixture of 45 % soil-45 % cattle manure and 10 % zeolite), but significantly higher than T3 (mixture of 90 % soil and 10 % zeolite). These results indicate the

Table 2. Effect of substrate type on the acclimatization of *in vitro* mulberry plants 30 days after planting.

Treatment	Survival, %	Length, cm	Number of leaves/plant	Leaf length, cm	Leaf width, cm
T1	87,5	7,4[a]	6,7[a]	4,4[a]	3,2[a]
T2	88,6	7,1[a]	6,5[a]	4,4[a]	3,1[a]
T3	85,3	5,4[b]	5,3[b]	3,4[b]	2,6[b]
SE ±	2,36	0,080	0,090	0,100	0,060

Means with different letters in the same column differ statistically according to Tukey for $p \leq 0,05$.

beneficial effect of the inclusion of cattle manure in these mixtures, which could be associated to the improvement of the physical properties of the substrate and the content of nutrients and organic matter that is achieved with the addition of manure.

Similar results in the variable survival were reported by Salas *et al*. (2011), who found values higher than 90 % in *M. alba* vitroplants from temporary immersion systems and from semisolid culture media in different substrates and substrate mixtures composed by zeolite and earthworm humus.

In this regard, Clapa *et al*. (2015) reported 90 % survival in *Morus nigra* L. vitroplants when mixtures of commercial solid substrates were used. However, in a study for the acclimatization of *in vitro* plant of this species, in which a mixture of soil, vermicompost and sand (1:1:2) was used, Gogoi *et al*. (2017) observed severe wilting and low survival (40 %) when the plants were directly transferred to the greenhouse, which was ascribed to the little development of the stomas and epicuticular wax, causing water loss on the leaf surface.

On the other hand, Indacochea-Ganchozo *et al*. (2017) evaluated the acclimatization of three endangered forestry species [*Myroxylon balsamum* (L.) Harms, *Tabebuia crhysantha* and *Tabebuia billbergii* (Bureau & K.Schum.) Standl.], using a substrate composed by 40 % river sand, 40 % earthworm humus and 20 % sawdust from decomposed timber. The acclimatization of the vitroplants of the three species was achieved in a ten-week period, with a survival of 65, 80 and 70 %, respectively; and the vitroplants reached a size between 17,07 and 19,53 cm and a number of leaves that varied between 7 and 14 per plant.

Effect of substrate type on the growth of vitroplants nursery conditions

In most crops, the plants obtained by tissue culture require to be transplanted into polyethylene bags until the vitroplants reach the quality requirements to be planted under production conditions.

The variables plant length and number of leaves per plant were significantly higher in T1 and T2, compared with T3 (table 3); while the variables leaf length and width did not show significant differences among the treatments. The results could be given by the inclusion of cattle manure in substrates T1 and T2, which contributes to improve the physical and chemical properties of soils and is a source of organic matter and nutrients that favors plant growth.

In a study conducted by Vílchez *et al*. (2015), the growth in nursery of seedlings and vitroplants of guava tree cv. Enana Roja Cubana EEA-1840 was compared, on a substrate composed by a mixture of fertilizer and washed river sand in proportion 3:1. During 70 days after the transplant, the physiological components of growth were evaluated fortnightly, such as: dry matter accumulation, crop growth rate, relative growth index, net assimilation rate, leaf area, number of leaves and stem and root length. During the first 28 days after transplant, the seedlings surpassed vitroplants in dry matter accumulation and growth; but later and until the end of the evaluation period, the vitroplants surpassed seedlings in the evaluated indicators. Such results coincide with the ones obtained in this work, where a growth and development of vitroplants was observed as the acclimatization time passed; at the end of the evaluation period normal and vigorous plants were obtained, with the adequate size to be planted in the field.

On the other hand, Adriano-Anaya *et al*. (2013) studied the use of compost during the acclimatization stage of vitroplants of banana tree clone Gran Enano (*Musa AAA*) in polyethylene bags. The results indicated that the plant growth was inhibited in the substrates with more than 30 % compost, which was ascribed to a possible effect of phytotoxicity caused by the chemical characteristics of planting substrates, for which the authors assumed that the salt content in the substrates could have been the factor that limited plant growth. In this re-

Table 3. Influence of substrate type on the growth of mulberry vitroplants under nursery conditions.

Treatment	Length, cm	Number of leaves/plant	Leaf length, cm	Leaf width, cm
1	20,7[a]	5,6[a]	10,0	7,1
2	18,9[a]	5,5[a]	9,9	7,3
3	12,7[b]	4,6[b]	9,7	6,9
SE ±	0,780	0,130	0,190 n.s	0,130 n.s

Means with different letters per column significantly differ according to Tukey's test for $p < 0,05$.

search the substrates did not show phytotoxicity in any of the used combinations.

It is concluded that the use of *in vitro* plants with a size between 1,5 and 2,5 cm and mixtures of substrates composed by soil cattle manure and zeolite in a ratio 70-20-10 % and 45-45-10 % during the acclimatization allowed to reach high survival values and higher growth and development of *M. alba* plants.

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