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ARTÍCULO ORIGINAL

In vitro micrografting of *Theobroma cacao* (Malvaceae) genotypes

Microinjerto in vitro *de genotipos de* Theobroma cacao *(Malvaceae)*

Yulien Miguelez-Sierra[1*], Adileida Mengana-Fresco[1], Pablo Clapé-Borges[2], Wilfredo Lambertt -Lobaina[2], Annia Hernández-Rodríguez[3*]

1 Faculty of Agronomy and Forestry, Universidad de Guantánamo, Cuba

2 Instituto de Investigaciones Agroforestales UCTB Baracoa, Cuba.

3 Department of Microbiology and Virology, Faculty of Biology, Universidad de La Habana, Cuba

*Autoras para correspondencia:
yulien@cug.co.cu
annia@fbio.uh.cu

ABSTRACT

The *in vitro* culture of *Theobroma cacao* has been limited due to the poor shoot growth and rooting which is why the species is considered recalcitrant in tissue culture. Bud micrografting could be an alternative in obtaining plants by biotechnological methods. The aim of the present study was to evaluate the *in vitro* grafting of different cacao genotypes and acclimatization of plants obtained by micrografting method. Cuban traditional cacao genotypes were used as scions and commercial genotypes were the rootstocks. The grafting method was side graft with apical bud from young plants. Success rates ranging 61-86.6 % and growth of buds were observed in micrografts of three traditional genotypes onto the same rootstock until the week nine. A similar response (91-100 %) was obtained in different combinations scion-rootstock and there were not significant differences in the responses of the genotypes to the micrografting. Acclimatization of micrografted plants to *ex vitro* conditions was successful with survival percentages ranging 64-85 %. The micrografting of these genotypes allowed obtaining viable rooted plants as a complement to the traditional vegetative propagation with application in germplasm conservation of cacao.

Key words: acclimatization *ex vitro*, cacao, in vitro grafting, clonal propagation

RESUMEN

El cultivo in vitro *de* Theobroma cacao *presenta limitaciones en el crecimiento de las yemas y el enraizamiento de los brotes por lo que la especie es considerada recalcitrante en cultivo de tejidos. El microinjerto de yemas podría ser una alternativa para la obtención de plantas por vía biotecnológica. El objetivo de este trabajo es evaluar el injerto in vitro de diferentes genotipos de cacao y la aclimatización de plantas obtenidas mediante el método de microinjerto. Se utilizaron genotipos de cacao tradicional cubano para las yemas y genotipos comerciales como patrones en el injerto. El método de injerto fue lateral con yema apical de plantas jóvenes. El porcentaje de éxito del*

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injerto fue de 61-86,6 % y se obtuvo crecimiento de las yemas en los microinjertos de tres genotipos tradicionales con el mismo patrón hasta las nueve semanas de cultivo. La respuesta fue similar (91-100 %) en diferentes combinaciones de yema y patrón sin diferencias significativas en las respuestas de los genotipos al microinjerto. La aclimatización de las plantas microinjertadas a las condiciones ex vitro fue exitosa, con porcentajes de supervivencia de 64-85 %. El microinjerto de estos genotipos permitió la obtención de plantas viables y enraizadas que pueden ser un complemento en la propagación clonal y tener aplicación en la conservación de germoplasma de cacao.

Palabras clave: *aclimatización* ex vitro*, cacao, injerto* in vi*tro, propagación clonal*

INTRODUCTION

Micrografting is an *in vitro* grafting technique that consists of placing a meristem or bud on a decapitated rootstock that has grown from seed germination or *in vitro* microscions (Monteuuis, 2012). The micrografting technique has numerous applications such as diagnosis and elimination of viruses and viroids, production of virus and viroid resistant plants, graft incompatibility study, physiological analysis of rejuvenation, improvement of plant regeneration, mass multiplication and safe exchange of germplasm (Hussain *et al*., 2014; Badalamenti *et al*., 2016).

Cacao, like most tropical trees, is recalcitrant in tissue culture. Poor results are reported in the culture of apices, axillary buds and microshoots due to poor response in shoot regeneration and rooting (Figueira and Alemanno, 2004). To overcome these limitations, the technique of micrografting somatic embryos on *in vitro* germinated rootstocks was assessed, which allowed an 83.3 % grafting success rate after 10 months of culture (Aguilar *et al*., 1992). However, this method has the disadvantage that scions come from somatic embryogenesis in which the appearance of genetic variants in regenerated plants is frequent (Garcia *et al*., 2019; Henao-Ramírez *et al*., 2021). More recently, a micrografting method of apical and axillary buds of cacao onto *in vitro* germinated seedlings was developed obtaining a graft success ranging from 55 to 95 % (Miguelez-Sierra *et al*., 2017). The *in vitro* micrografting of buds could be an alternative for the clonal propagation of promising genotypes of *Theobroma cacao*and their conservation in safer conditions taking into account the vulnerability of *in situ* collections exposed to climatic events and pests that highly affect the crop. On the other hand, rooted plants, suitable for transfer to soil would be obtained, which could help to overcome the difficulties in the growth of isolated buds and the rooting of shoots, with the additional advantage that grafting is also used to produce elite plants in cacao-producing regions. In this study, the *in vitro* grafting of different cacao genotypes and acclimatization of plants obtained by micrografting method were assessed.

MATERIALS AND METHODS

Plant material, seedlings preparation and culture conditions

Plant material from the Instituto de Investigaciones Agroforestales (INAF), UCTB Baracoa, Guantánamo, Cuba was used. Commercial genotypes UF 677, UF 613 and IMC 67 (International Cocoa Germplasm Database [ICGD], 2019) commonly used as rootstock in the production system of plants and traditional Cuban cacao genotypes identified as Trinitario (Bidot *et al*., 2015) were used. The traditional genotypes are included in the core collection for studying the genetic diversity of the Cuban ancient cacao.

Seeds extracted from mature fruits of trees aged five-six years were used. Seeds were disinfected with a calcium hypochlorite solution 2 % (w/v) for 20 min and then washed three times with sterile distilled water under aseptic conditions. After removing the mucilage from the seeds, one third of the seeds were cut at the opposite end of the embryo position. The seeds were then placed in test tubes containing 10 mL of Murashige and Skoog (MS) culture medium (Murashige and Skoog, 1962), supplemented with sucrose 30 g L^{-1}, adjusted to pH 5.7 and solidified with Plant Agar (Duchefa, Biochemie) 6 g L^{-1}.

In vitro cultures were maintained in growth chamber at a temperature of 25 ± 1°C, relative humidity 60 %, 23 µmol m^{-2} s^{-1} (photosynthetic photon flux) and photoperiod 16 h/8 h (light/dark).

Micrografted plants were obtained using the protocol described by Miguelez-Sierra *et al*. (2017) for the *in vitro* micrografting of *T. cacao* using side graft with apex from young plants. Apexes from four-month-old plants were side grafted on hypocotyls of three-week-old seedlings obtained *in vitro*. The apex is inserted in a lateral incision (30º angled) and the grafted area is covered with a gel made of low melting agarose (2 %). Micrografted plants were planted in MS medium supplemented with sucrose (3 %), pH 5.7 and solidified with Plant Agar (5 g L^{-1}). Culture vessels of 1-L capacity were filled with 250 mL of medium. Cultures were maintained in the same conditions as above.

In vitro micrografting of different genotypes

The traditional cacao genotypes EICB-371, EICB-384 and EICB-385 were used as a source of apical buds that were grafted onto rootstocks of genotype UF 677. The development of the micrografts was evaluated weekly from the first week of culture until week nine. Survival percentage, grafting success percentage and number of leaves were evaluated at week nine. Thirty, 26 and 32 grafts were made for genotypes EICB-371, EICB-384 and EICB-385, respectively.

***In vitro* micrografting with different scion-rootstock combinations**

Traditional cacao genotypes L-270, L-276, L-280 and K-251 were used as scion source and grafted onto rootstocks of genotypes IMC 67 and UF 613. At nine weeks, survival percentage, grafting success percentage and number of leaves were evaluated.

Acclimatization of micrografted plants

The experiment was performed in order to evaluate the micrografting method in obtaining viable plants able to survive and growth in *ex vitro* conditions. For that purpose, micrografted plants nine-week-old with buds of EICB-371, EICB-384 and EICB-385 genotypes onto UF 677 rootstocks were used. Plants were removed from the culture containers for transfer to the acclimatization stage. Remnants of culture medium were removed from the roots with plenty of distilled water and plants were placed in trays with distilled water until transplanting. The grafts were placed in 1 L plastic pots with Vivimus DCM® Universal substrate. Plants were maintained for two weeks in chambers with high humidity (100 %), photoperiod 16 h/8 h (light/dark), 25-26ºC and illumination of 119.85 µmol m^{-2} s^{-1} photosynthetic photon flux (4:1 white and yellow light provided by Agrosor 400 W lamps).

Irrigation was applied three times a week with 100 mL of water to each plant. At four weeks, survival, number of leaves and shoot length were evaluated.

Experimental design and statistical analysis of data

The experiments were carried out according to a completely randomized design and were repeated three times. Data on number of leaves and shoot length did not conform to normality and homogeneity of variance and were analyzed with Kruskal-Wallis ANOVA by ranks. Data expressed as percentages (survival and graft success) were analyzed by comparison of proportions. In all cases a 95 % confidence interval was used. Data analysis was performed with Statistica 8.0.360.

RESULTS

In vitro micrografting of different genotypes

Micrografting of apexes of traditional Cuban genotypes on UF 677 rootstocks provided information on the dynamics of bud growth during nine weeks (Fig. 1). Survival was 100 % in all three genotypes up to week five and at week six 93.3 % in EICB-371, 84.7 % in EICB-384 and 75 % in EICB-385. These values remained constant until week nine and had no significant differences (Fig. 1a).

Grafting success percentages at week nine were 61.5-86.6 % with no significant differences between genotypes (Fig. 1b). These values were achieved gradually from bud break over the weeks. The shoot growth expressed in the number of leaves, had a progressive increase for the three genotypes. At nine weeks of culture, an average of 3.75 leaves per shoot was obtained for genotype EICB-385, 3.20 for genotype EICB-371 and 2.15 for genotype EICB-384 (Fig. 1c) with no significant differences.

The results show that micrografted plants of the genotypes EICB-371, EICB-384 and EICB-385 onto UF677 rootstock maintained continuous growth of shoots since the second week of culture until week nine (Fig. 2). In the second week, formation of a callus bridge between rootstock and scion tissues was evident and consequently shoots started growing indicating a well union formation in successful grafts (Fig. 2a). After three weeks, leaves development and shoot elongation was observed allowing plants to continue growing in successive weeks in all the three genotypes (Fig. 2 b,c).

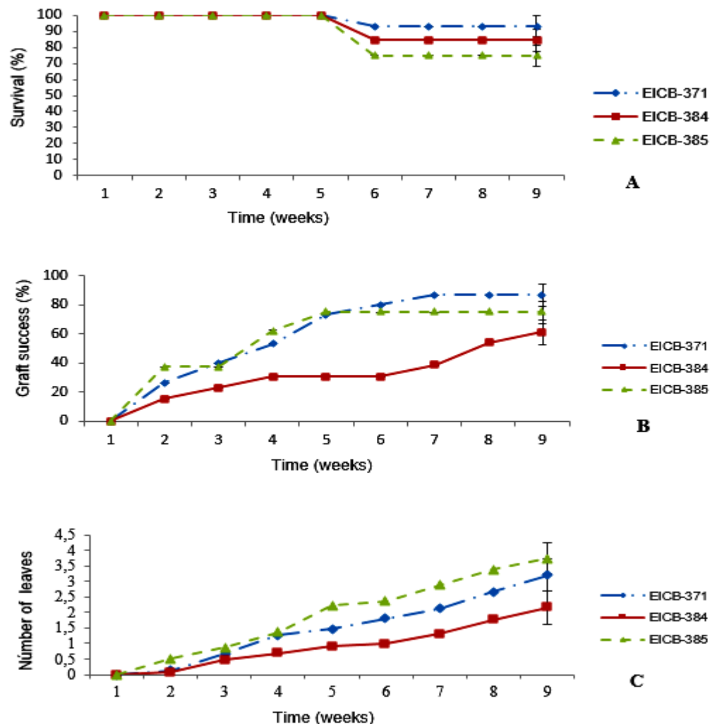

Figure 1. Response of traditional Cuban cacao genotypes (EICB-371, EICB-384 and EICB-385) to *in vitro* micrografting of apex with UF 677 rootstock during nine weeks of culture. A) Survival, B) Grafting success and C) Number of leaves. Survival and grafting success were analyzed by comparison of proportions and number of leaves with Kruskal-Wallis ANOVA by ranks at week nine. There were no significant differences at p<0.05. Vertical bars represent the standard error of the mean.

Figura 1. *Respuesta de genotipos tradicionales de cacao cubano (EICB-371, EICB-384 y EICB-385) al microinjerto* in vitro *de ápice con portainjerto UF 677 durante nueve semanas de cultivo. A) Supervivencia, B) Éxito de injerto y C) Número de hojas. La supervivencia y el éxito del injerto se analizaron por comparación de proporciones y número de hojas con Kruskal-Wallis ANOVA por rangos en la semana nueve. No hubo diferencias significativas a p<0,05. Las barras verticales representan el error estándar de la media.*

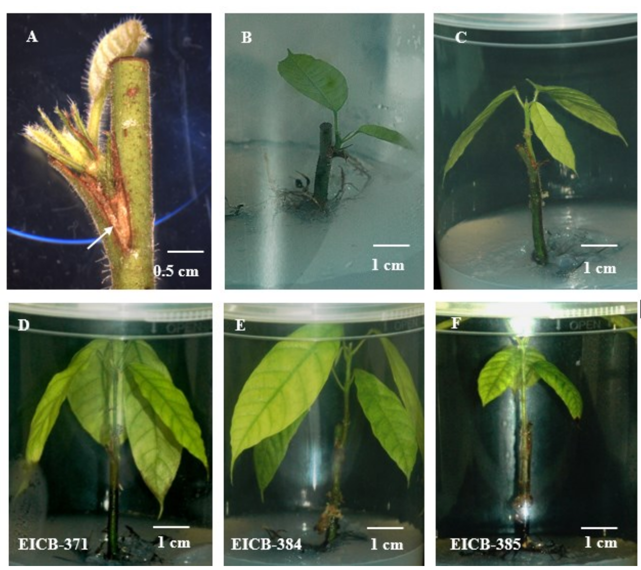

Figure 2. *In vitro* micrografted plants with apex side grafted on rootstocks of genotype UF 677. A, B and C, micrografts of traditional Cuban genotype EICB-371 at two, three and six weeks of age, respectively. D, E and F, micrografts of genotypes (EICB-371, EICB-384 and EICB-385, respectively), at nine weeks of age. Arrow indicate the callus bridge between rootstock and scion tissues.

Figura 2. *Plantas microinjertadas* in vitro con injerto lateral de ápice *sobre portainjertos del genotipo UF 677. A, B y C, microinjertos del genotipo cubano tradicional EICB-371 a las dos, tres y seis semanas de edad, respectivamente. D, E y F, microinjertos de genotipos (EICB-371, EICB-384 y EICB-385, respectivamente), a las nueve semanas de edad. La flecha indica el puente del callo entre el portainjerto y los tejidos del brote.*

In vitro micrografting with different scion-rootstock combinations

Micrografts of genotypes L-270, K-251, L-280 and L-276 on rootstocks of genotypes UF 613 and IMC 67 had no significant differences in terms of survival, graft success and number of leaves (Table 1).

Table 1. Survival, grafting success and number of leaves obtained with different combinations of rootstock and scion genotypes in the *in vitro* micrografting of *T. cacao* at week nine in culture. Means ± standard error are presented. The analysis of data expressed as percentages was performed by comparison of proportions. Leaf number data were analyzed with Kruskal-Wallis ANOVA by ranks. There were no significant differences at p<0.05. ns- no significant.

Tabla 1. *Supervivencia, éxito de injerto y número de hojas obtenidas con diferentes combinaciones de genotipos de portainjertos y yemas en el microinjerto* in vitro *de* T. cacao *en la semana nueve de cultivo. Se presentan las medias ± error estándar. El análisis de los datos expresados en porcentajes se realizó por comparación de proporciones. Los datos del número de hojas se analizaron con Kruskal-Wallis ANOVA por rangos. No hubo diferencias significativas a p<0,05. ns- no significativo*

Genotype scion/ rootstock combinations	Number of grafts	Survival %	Graft success %	Number of leaves
L-270/UF 613	26	100±0	84.6±7.7	2.09±0.21
L-270/IMC 67	22	100±0	81.8±9.1	2.44±0.33
K-251/UF 613	22	91.7±5.89	83.3±8.3	2.30±0.30
K-251/IMC 67	20	100±0	60±14.1	1.83±0.30
L-280/UF 613	20	100±0	90±7.1	2.11±0.20
L-280/IMC 67	20	100±0	80±10	3.12±0.35
L-276/UF 613	20	100±0	90±7.1	2.00±0.23
L-276/IMC 67	20	100±0	80±10	2.75±0.36
		ns	ns	ns

Acclimatization of micrografted plants

Micrografts of three traditional cacao genotypes on UF 677 rootstocks had a survival between 64-85 % at four weeks in the acclimatization phase (Table 2). There were no significant differences in survival, number of leaves and shoot length. This response is similar to the *in vitro* growth response of grafted apexes of these genotypes where there were no significant differences between genotypes.

Table 2. Survival and growth in acclimatization phase of micrografted plants of Cuban traditional cacao genotypes on UF 677 rootstock at 4 weeks of culture. Means ± standard error are presented. The analysis of survival percentage was performed by comparison of proportions. Shoot length and number of leaves were analyzed with Kruskal-Wallis ANOVA by ranks. There were no significant differences at p<0.05. ns- no significant .

Tabla 2. *Supervivencia y crecimiento en fase de aclimatación de plantas microinjertadas de genotipos tradicionales de cacao cubano sobre portainjerto UF 677 a las 4 semanas de cultivo. Se presentan las medias ± error estándar. El análisis del porcentaje de supervivencia se realizó por comparación de proporciones. La longitud de los brotes y el número de hojas se analizaron con Kruskal-Wallis ANOVA por rangos. No hubo diferencias significativas a p<0,05. ns- no significativo*

Genotypes	Number of grafts	Survival %	Number of leaves	Shoot length (cm)
EICB-371	14	64.3±15.9	7.11±0.88	3.87±0.27
EICB-384	14	85.7±10	8.92±0.62	3.99±0.8
EICB-385	14	64.3±15.9	6.67±0.49	4.21±0.4
		ns	ns	ns

Shoot development on the micrografted plants during the four weeks of acclimatization was prominent as they emitted several pairs of leaves for a total of 6-9 leaves per plant, compared to 2-3 leaves they had when transferred. Shoot length also experienced a continuous increase to 3-4 cm.

DISCUSSION

In this study, several genotypes of Cuban traditional cacao had similar responses to micrografting of apex onto rootstocks from seedlings obtained *in vitro*, probably due to the genetic similarity of these genotypes of cacao Trinitario natives from Baracoa (Cuba). Bidot *et al*. (2015) report that plants of ancient cacao growing in the Cuban Eastern have in common a higher proportion of the genetic groups Criollo, Amelonado and Marañón respect the other genetic groups. This genetic proximity can influence the physiological response to micrografting in the genotypes. Consistent with these results, it has been observed that different cultivars of a species could have similar establishment and growth response to grafting, i.e., Iranian grapevine (*V. vinifera*) cultivars Sahebi, Soltanin and Fakhri (Aazami and Hassanpouraghdam, 2010) and almond cultivars

Texas, Ferrastar and Nonpareil (Yıldırım *et al*., 2013). Also, was evident that apexes of the different genotypes had a good response in micrografting with the same rootstock, in this case UF 677, indicating the existence of compatibility between these genotypes and the rootstock, which is also Trinitario type. In this regard, Hsina and Mtili (2009) and Davoudi *et al*. (2019) report a high level of compatibility and high regeneration rates in micrografting with bud and rootstock of the same species, in *Ceratonia siliqua* L. and *Rosa* genus, respectively. Estrada-Luna *et al*. (2002) state that the evidence of graft compatibility is the establishment of vascular continuity in the tissue and the high percentage of grafting success expressed as bud growth. Nevertheless, incompatible grafts are characterized for poor vascular connection and phloem degeneration in the junction zone a few weeks after graft establishment what produce non sprouting buds (Hussain *et al*., 2014).

On the other hand, the in *vitro* grafting of several genotypes of traditional cacao on two different rootstocks was successful indicating a high level of compatibility between these genotypes of Trinitario type. These results suggest the possibility of applying the micrografting method to different combinations scion-rootstock with closely related genetic traits. The micrografting of the same type of bud on rootstocks of different cultivars has been successful in various species. Apices of *V. vinifera* variety Early Cardinal grafted on hypocotyls of American rootstocks Salt Creek and 41 B had success rates of 71.4 % and 81 %, respectively (Tangolar *et al*., 2003). Singh *et al*. (2019) successfully grafted buds of *C. reticulata* Blanco onto rootstocks of cultivars Nemutenga, Tayum, and Tasi. Nevertheless, Asadi and Shekafandeh (2021) reported differences in grafting success of scions of almond cultivar Sahand on two wild rootstocks (Badam Kohi and Arjan) that was related with some metabolites produced during the union formation.

During acclimatization of cacao micrografted plants, the effects observed in the survival response were due to the variation in the growing conditions experienced by the plants in the transfer to the *ex vitro* environment. It is known that transplanting and establishment of plants produced *in vitro* to greenhouse requires the adaptation of plant physiology, and many of them die in this process. Several authors report similar survival values (65-80 %) that they considered successful in the acclimatization of forest species obtained *in vitro*. Niang *et al*. (2010) on micrografted plants of *Sterculia setigera* Del. Indacochea-Ganchozo *et al*. (2017) on *Myroxylon balsamum* (L.) Harms, *Tabebuia crhysantha* (Jacq.) and *Tabebuia billbergii* (Bureau and K. Scum.) Standl. Also, Ferrara *et al*. (2015), on *Lippia rotundifolia* Cham. plants, at two weeks in acclimatization.

Similarly, successful acclimatization of micrografted plants was described by Mneney and Mantell (2001) in *Anacardium occidentale* L., Singh *et al*. (2008) in *Citrus nobilis* Lour x *C. deliciosa* Tenora and Arif *et al*. (2009) in *Pisum sativum* L.

The success obtained in the *in vitro* micrografting of cacao genotypes from the applied method evidences that it is possible to generate grafted plants by this way. This method could be useful in the study of compatibility in the grafting of traditional Cuban cacao genotypes on different rootstocks. The method could also be applied to other cacao genotypes with bud-rootstock combinations used in traditional propagation by grafting.

In this research, progress has been made in the *in vitro* grafting of different cacao genotypes and acclimatization of plants obtained by micrografting method. The high percentage of success and continuous shoot growth obtained in the micrografting of several genotypes of Cuban traditional cacao indicate that this method has potential for overcoming the limitations of *in vitro* culture of buds in the species. The response of the genotypes in the establishment of micrografts *in vitro* was similar in all scion-rootstock combinations what evidences the existence of graft compatibility between these genotypes of cacao Trinitario and the possibility of applying the micrografting to different genotypes. Micrografted plants of three different genotypes continued growing under *ex vitro* acclimatization conditions suggesting that the performed micrografting method provides viable plants suitable for transferring to soil. The micrografting of cacao apexes from young plants allowed to obtain rooted and vigorous plants as an alternative to the traditional clonal propagation and germplasm conservation.

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