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Original article

Optimization of *in vitro* disinfection and establishment of *Tithonia diversifolia* ICACuba Oc-10

Optimización de la desinfección y establecimiento *in vitro* de *Tithonia diversifolia* ICACuba Oc-10

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ABSTRACT: Disinfection and *in vitro* establishment are critical stages in tissue culture. In *Tithonia diversifolia*, the response to these phases is unknown; therefore, the objective of the following study was to optimize surface disinfection with sodium hypochlorite (NaClO) and the *in vitro* establishment of apical buds of *T. diversifolia* ICACuba Oc-10. The research was carried out at the Plant Biotechnology Laboratory of the Faculty of Agronomy at the Agrarian University of Havana, using apical buds of *T. diversifolia* ICACuba Oc-10 as plant material for *in vitro* implantation. The experiment followed a completely randomized design with four treatments consisting of 1, 4, and 6 % NaClO, and a control with sterile distilled water. At seven days, the variables percentage of total contaminated explants, explants contaminated with fungi and bacteria were evaluated, and sprouting was assessed at ten days during the *in vitro* establishment phase. The 6 % NaClO concentration was most effective for surface disinfection of apical buds of *T. diversifolia* ICACuba Oc-10 and for eliminating fungal and bacterial contamination at seven days of *in vitro* establishment. Fungal contamination was found in all treatments with NaClO concentrations below 6 %; however, bacterial contamination only occurred in the control. No explant sprouting was observed at ten days, suggesting the need for a longer establishment period.

Key words: contamination, micropropagation, sodium hypochlorite.

RESUMEN: La desinfección y establecimiento *in vitro* son etapas críticas en el cultivo de tejidos. En *Tithonia diversifolia* se desconoce su respuesta ante estas fases, por lo que el objetivo del siguiente estudio fue optimizar la desinfección superficial con hipoclorito de sodio (NaClO) y el establecimiento *in vitro* de yemas apicales de *T. diversifolia* ICACuba Oc-10. La investigación se desarrolló en el Laboratorio de Biotecnología Vegetal de la Facultad de Agronomía en la Universidad Agraria de La Habana y se utilizaron yemas apicales de *T. diversifolia* ICACuba Oc-10 como material vegetal para implantar *in vitro*. El experimento presentó un diseño completamente al azar con cuatro tratamientos compuestos por uno, cuatro y 6 % de NaClO y un control con agua destilada estéril. Se evaluaron, a los siete días, las variables porciento de explantes contaminados totales, explantes contaminados con hongos y bacterias y la brotación a los diez días en la fase de establecimiento *in vitro*. La concentración de NaClO al 6 % fue más efectiva en la desinfección superficial de yemas apicales de *T. diversifolia* ICACuba Oc-10, y en la eliminación de la contaminación por hongos y bacterias a los siete días de establecimiento *in vitro*. La contaminación por hongos se encontró en todos los tratamientos con concentraciones de NaClO inferiores al 6 %, sin embargo, la contaminación bacteriana solo ocurrió en el control. No se observó brotación de los explantes a los diez días, sugiriendo la necesidad de mayor tiempo de establecimiento.

Palabras clave: contaminación, hipoclorito de sodio, micropropagación.

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INTRODUCTION

Tithonia diversifolia (Hemsl.) A. Gray is a shrub of the Asteraceae family that, due to its ability to adapt to multiple environmental, edaphic, and management conditions, its regrowth capacity and rapid growth, as well as its high nutritional value and contribution, has demonstrated its potential for animal feed. It propagates through sexual and vegetative seeds. Furthermore, its establishment in silvopastoral systems and its use as green manure can improve soil fertility conditions to increase pasture production and yield. These characteristics give it high potential for cattle feed, either as a browsing resource, processed into flour or silage, reducing production costs in livestock systems (1,2).

T. diversifolia ICACuba Oc-10 accession is the result of the study of a group of *T. diversifolia* shrub materials collected in the Central-West region of Cuba to develop a technology with those most outstanding for biomass production, response to cutting and grazing, as well as their evaluation in biological and physiological parameters of animals used for livestock development. It has the capacity to produce 13.1 and 3.67 t DM·ha^{-1}·year^{-1} as forage or under grazing, respectively, with 17 % of that in the dry season. Its foliage contains 19.7 % crude protein, 36.24 % neutral detergent fiber, 15.48 % acid detergent fiber, and 73.2 % DM digestibility. In addition, it reduces methanogenic microorganisms in the rumen, reaching values of 16.2 × 10^{9} CFU·mL^{-1}, which helps mitigate methane emissions into the atmosphere (3).

On the other hand, one of the main problems in micropropagation is the presence of endogenous microbial contaminants that can cause the death of plant tissues, as they compete for nutrients and modify the culture medium (4). In this regard, the surface disinfection of explants and the concentration of the disinfectant can be decisive for the success of this technique, and among the most widely used substances is sodium hypochlorite (NaClO), due to its low cost, easy acquisition, lower phytotoxic effect on tissues, and low negative impact on operator health (5,6).

Currently, in the available literature, there are no studies on the stages of *in vitro* culture of *T. diversifolia*, therefore, the optimization of procedures for the disinfection and *in vitro* establishment stages is essential to initiate micropropagation, genetic improvement, and germplasm conservation studies of this important forage shrub. Based on these antecedents, the objective of the following study was to optimize the surface disinfection with sodium hypochlorite and the *in vitro* establishment of apical buds of *T. diversifolia* ICACuba Oc-10.

MATERIALS AND METHODS

The research was carried out at the Plant Biotechnology Laboratory of the Faculty of Agronomy at the Agrarian University of Havana "Fructuoso Rodríguez Pérez" (UNAH), located in the municipality of San José de Las Lajas, Mayabeque province.

Plant material

Apical buds of the *T. diversifolia* ICACuba Oc-10 accession were taken, all with the same regrowth age, established in the seed bank of the "Miguel Sistachs Naya" Experimental Center for Pastures and Forages of the Institute of Animal Science, San José de las Lajas, Mayabeque, Cuba, located at 22°53' N and 82°02' W at 80 m above sea level.

Collection and transport

Collection was carried out in the morning (8:00 to 10:00 a.m.). Gardening shears were used to cut the apical buds, which were then placed in a flask containing distilled water and the antioxidants citric acid at 150 mg L and ascorbic acid at 100 mg L to prevent plant tissue browning. After collection, the explants were transported at room temperature to the Biotechnology laboratory as quickly as possible.

Disinfection before laminar flow

In the laboratory, the apical buds were rinsed with distilled water and then washed with commercial detergent under agitation for 5 minutes, followed by three rinses with distilled water.

Disinfection in laminar flow

The laminar flow cabinet was properly disinfected with 70 % ethanol, and an ultraviolet lamp was turned on for 30 minutes before starting the planting. The explants were disinfected with 70 % ethanol for one minute, then rinsed three times with sterile distilled water, and three concentrations of sodium hypochlorite were tested during a five-minute immersion. Finally, they were rinsed with sufficient sterile distilled water before planting.

Planting and *in vitro* establishment

The apical explants were placed in flasks containing 25 mL of Murashige and Skoog basal culture medium (7) with 4.42 g L, sucrose at 30 g L, 1 mg L 6-benzylaminopurine (6-BAP), 2 mg L naphthaleneacetic acid (NAA), 2 mg/L indole-3-acetic acid (IAA), and solidified with agar at 10 g L. To prevent medium browning, the antioxidants citric acid at 150 mg L and ascorbic acid at 100 mg L were added. The pH was adjusted to 5.7 with 0.1N sodium hydroxide and 0.1N hydrochloric acid before sterilization.

The media were sterilized in an autoclave for 20 minutes at 121 °C and one atmosphere of pressure, and stored at room temperature for 72 hours before planting. Prior to implantation, the utensils were sterilized in an autoclave for 30 minutes at 121 °C and one atmosphere of pressure. The experiment was established in a growth chamber at a temperature of 25 ± 2 °C, relative humidity of 80-90 %, artificial light with a photoperiod of 16 hours of light and light intensity of 18.75 µmol·m^{-2}·s^{-1}.

Treatments and experimental design

A completely randomized design was used with four treatments consisting of 1, 4C, and 6 % NaClO, and a control with sterile distilled water. Each treatment had three replicates, with three explants established per experimental unit (glass flasks).

Variables analyzed

After seven days of *in vitro* establishment, the variables percentage of total contaminated explants, number of explants contaminated with fungi, number of explants contaminated with bacteria were evaluated, and sprouting was assessed at ten days.

Statistical analysis

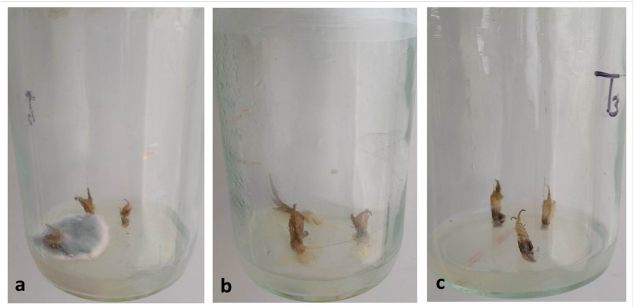
A proportion comparison analysis (chi-square) was performed, and Fisher's exact test was used for $p < 0.05$ (8) for the variable number of explants contaminated with fungi. The data were analyzed using the statistical package ComparPro version 1 (9).

RESULTS AND DISCUSSION

The behavior of the percentage of total contaminated explants showed, at seven days, that the control treatment without the use of sodium hypochlorite presented contamination by fungi and bacteria in all of the *T. diversifolia* ICACuba Oc-10 explants (Figure 1A and 1B). Bacterial contamination was only visible in the control treatment and was not observed in the treatments where different NaClO concentrations were used. The treatment with 6 % sodium hypochlorite showed the best response, as it presented no microbial contamination (Figure 1C). The latter demonstrated greater effectiveness in reducing contamination, suggesting that NaClO at this concentration is more effective for explant disinfection.

In treatments with sodium hypochlorite concentrations below 6 %, fungal contamination predominated, reaching up to 33.33 % in the control treatment and in the 1 % NaClO treatment (Table 1), in contrast to the low incidence of bacterial contamination, which was only observed in the control treatment. On the other hand, at ten days of culture, shoot formation was not observed in any of the evaluated treatments, which could indicate that the explants require more time in the establishment phase to begin sprouting apical buds.

Effective disinfection occurs when explants have been exposed to low concentrations of disinfectant, show reduced rates of oxidation and microbial contamination, and allow shoot development (10). In this sense, the concentration of sodium hypochlorite should be between 1 and 3 % to activate cell differentiation of the explants and achieve good disinfection (11). On the other hand, concentrations above 3 % sodium hypochlorite cause phytotoxic damage and explant tissue death (10). This approach contrasts with the results obtained in the present study, which demonstrate that 6 % sodium hypochlorite can be used with a 5-minute immersion without causing the death of *T. diversifolia* explants.



a. Explants contaminated with fungi in the control treatment. b. Explants contaminated with bacteria in the control treatment. c. Treatment with 6 % sodium hypochlorite, showing no microbial contamination

Figure 1. Apical explants of *T. diversifolia* ICACuba Oc-10 after seven days of *in vitro* establishment

Table 1. Number of explants contaminated with fungi after seven days of *in vitro* establishment of *T. diversifolia* ICACuba Oc-10

Concentrations NaClO (%)	Number of explants contaminated with fungi	%	SE and Signif.
0	3	33.33	±13.19
1	3	33.33	p>0.05
4	1	11.11	
6	0	0	

Authors' own elaboration

Differences in contamination percentage are largely due to the surface action of sodium hypochlorite as a disinfecting agent and to the endogenous presence of contaminating microorganisms in the tissues (12). Endogenous contamination is one of the main causes of explant loss during the *in vitro* establishment stage, especially if it is of bacterial origin, as contamination rates above 90 % can be reached when 1 % sodium hypochlorite has been used as a disinfectant (13).

Obtaining contaminant-free explants is one of the critical points for the development of efficient micropropagation protocols, due to the presence of pathogens that contaminate the tissues. Microbial contamination is one of the serious problems limiting the successful extrapolation of plant tissue culture practices to other plant species. Sources of *in vitro* contamination include culture vessels, media, explants, the culture room, the transfer area, and operational personnel (14). It is important to note that the more intense the disinfection protocol, the more contamination is reduced, but the explants are severely damaged. The stress caused by disinfection could affect and prolong the establishment phase and the sprouting of the explants.

Several studies have shown that the use of disinfectant agents at high concentrations, such as 10 % sodium hypochlorite, can effectively eliminate the microbial load but also induces oxidation and necrosis in plant tissues,

compromising their regeneration. In this regard, two investigations observed that as the concentration and immersion time in sodium hypochlorite increase, the contamination percentage and survival rate decrease, while the percentage of necrosis or death of primary explants cultured *in vitro* increases (15,16).

CONCLUSIONS

The 6 % sodium hypochlorite concentration was most effective for the surface disinfection of apical buds of *T. diversifolia* ICACuba Oc-10 and for eliminating fungal and bacterial microbial contamination after seven days of *in vitro* establishment. No explant sprouting was observed at ten days, suggesting the need for a longer establishment period.

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