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Protocolo para desenvolvimento de embriões zigóticos *in vitro* e aclimatização de plantas de Sangue-de-dragão

Protocol for development of zygotic embryos *in vitro* and acclimatization of Dragon's Blood plantlets

Protocolo para el desarrollo de embriones cigóticos *in vitro* y aclimatación de plántulas de Sangre de dragón

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João Bosco de Oliveira Júnior
ORCID: https://orcid.org/0000-0002-9936-145X
Universidade Federal do Acre, Brasil
E-mail: junior.jboj@gmail.com
Camilla Mendes Pedroza Pessoa
ORCID: https://orcid.org/0000-0003-1947-1454
Universidade Federal do Acre, Brasil
E-mail: kmillampp@gmail.com
Hendril da Silva Lopes
ORCID: https://orcid.org/0000-0001-7674-061X
Universidade Federal do Acre, Brasil
E-mail: hendrillopes2017@gmail.com
Frederico Henrique da Silva Costa
ORCID: https://orcid.org/0000-0003-0118-3438
Universidade Federal do Acre, Brasil
E-mail: fredericohenrique@yahoo.com.br
Jonny Everson Scherwinski Pereira
ORCID: https://orcid.org/0000-0001-6271-332X
Empresa Brasileira de Pesquisa Agropecuária, Recursos Genéticos e Biotecnologia, Brasil
E-mail: jonny.pereira@embrapa.br

Resumo

Sangue-de-dragão, espécie nativa da América do Sul, possui propriedades terapêuticas comprovadas cientificamente. Este estudo teve como objetivo desenvolver um protocolo para estabelecimento *in vitro* e aclimatização de plantas a partir de embriões zigóticos, uma vez

que pesquisas com a espécie são incipientes. Foram avaliados meios de cultura (MS, WPM, QL e N6), na ausência e presença de carvão ativado (2 g L^{-1}). Observou-se percentagem de embriões desenvolvidos entre 83% a 100% e influência da composição do meio para altura de plantas e número de folhas *in vitro*. As plantas foram aclimatizadas com 100% de sobrevivência, e para os tratamentos no cultivo *in vitro* apenas o meio de cultura influenciou o diâmetro e altura das plantas. As formulações WPM e QL sem carvão ativado são indicadas para o cultivo *in vitro* de embriões e aclimatização de Sangue-de-dragão. A metodologia proposta representa uma estratégia para reprodução, estudos fisiológicos e preservação da espécie.

Palavras-chave: Planta medicinal; Meio de cultura; Carvão ativado; *Croton lechleri*.

Abstract

Dragon's blood, native species from South America, has therapeutic properties scientifically proved. This study had the objective of developing a protocol for in vitro establishment and plantlets acclimatization from zygotic embryos, since researches with species are incipient. Culture media (MS, WPM, QL and N6) were assessed, without and with activated charcoal (2 g L^{-1}). It was observed the percentage of developed embryos from 83% to 100% and the effect of medium composition for plant height and leaf number *in vitro*. The plantlets were acclimatized with 100% of survival rate, and for treatments from *in vitro* culture only the culture medium influenced diameter and plant height. The WPM and QL formulations without activated charcoal are indicated for *in vitro* culture of embryos and acclimatization of Dragon blood. The suggested methodology represents an important strategy for reproduction, physiological study and preservation of species.

Keywords: Medicinal plant; Medium culture; Active charcoal; *Croton lechleri*.

Resumen

La sangre de dragón, especie nativa de América del Sur, tiene propiedades terapéuticas científicamente comprobadas. Este estudio tuvo el objetivo de desarrollar un protocolo para el establecimiento *in vitro* y la aclimatación de plántulas a partir de embriones cigóticos, ya que las investigaciones con especies son incipientes. Se evaluaron los medios de cultivo (MS, WPM, QL y N6), sin y con carbón activado (2 g L-1). Se observó el porcentaje de embriones desarrollados del 83% al 100% y el efecto de la composición del medio para la altura de la planta y el número de hojas *in vitro*. Las plántulas se aclimataron con una tasa de supervivencia del 100%, y para los tratamientos de cultivo *in vitro* solamente el medio de

cultivo influyó en el diámetro y la altura de la planta. Las formulaciones de WPM y QL sin carbón activado están indicadas para el cultivo in vitro de embriones y la aclimatación de sangre de dragón. La metodología sugerida representa una estrategia importante para la reproducción, el estudio fisiológico y la preservación de especies.

Palabras clave: Planta medicinal; Medio de cultivo; Carbón activado; *Croton lechleri*.

1. Introduction

Croton lechleri Mull. Arg. (Euphorbiaceae), popularly known as Dragon's blood, is a pioneer and heliophyta tree spread over South America. This species produces a red-coloured sap or latex which contains taspine an alkaloid (Montopoli et al., 2012), with anti-inflammatory, antibiotic and wound-healing properties (Alonso-Castro et al., 2012). However, the sap's extraction method, considered predatory, is performed by cutting down the plant and/or bark removal, causing irreversible damage. Moreover, the species breeds spontaneously starting from the germination of seeds and gathering of seedlings on cultivation areas and meadows, putting its survival at risk, since it competes against agricultural crops (Lopes et al., 2013).

For medicinal plants, the cultivation of tissues is applied and suggested (Morais et al., 2012; Oliveira et al., 2011) envisioning the production of certified and standardized raw material, free of phytopathogens and plague disseminated by conventional propagation methods (Mafia et al., 2012). Such aspects are crucial for clonal cleaning and conservation *in vitro* of exotic and native species (Machado et al., 2013). The culture *in vitro* of zygotic embryos is one of the main strategies for multiplication, domestication and preservation of germplasm in plant species, as a result of the embryo's juvenile nature and regenerative potential (Hu & Ferreira, 1998). Apart from reducing the time required for the attainment of new healthy specimens, it guarantees elevated development of *in vitro* embryos (Ebert et al., 2014) and allows for studies related to the embryo's physiology (Haslam & Yeung, 2011).

According to our knowledge, this is the first study on the *Croton* genus to utilize zygotic embryos as an explant. Considering the importance of *Croton lechleri* on the treatment of multiple symptomatologies, added to the predatory means of latex attainment; the objective of this study was to develop a protocol for *in vitro* establishment and acclimatization of *C. lechleri* plants originated from zygotic embryos.

2. Materials and Methods

This paper was an research of a quantitative nature, according to Pereira et al. (2018). The research was fulfilled at the Biotechnology and Plant Anatomy Laboratory and at the Experimental Area of Universidade Federal do Acre (UFAC), Rio Branco, AC. For the attainment of seeds and extraction of the zygotic embryo, infructescences were collected from grown trees at the Zoobotanical Park of UFAC. The fruits featured a greenish mesocarp, presence of floral remnants and protrusions on the locus junction points.

The seeds were sorted and the ones featuring black tegument were selected, and in laminar flow conditions, such seeds were disinfected in ethyl alcohol 70% (v/v) for 1 minute, and a sodium hypochlorite solution (NaOCl) (1,25% of active chlorine v/v), plus Tween-80 (one drop per 50 mL), for 15 minutes, followed by three rinses in autoclaved water. With the aid of a stereomicroscope, the zygotic embryos were extracted and immediately transferred to test tubes with the culture mediums.

The experimental design was entirely randomized and the culture mediums, in a factorial 2 x 4 scheme, represented by the presence (2,0 $g.L^{-1}$) and absence of active charcoal matched with four formulations of culture media. The experimental unit consisted of a test tube (25 mm x 150 mm) containing 10 mL of medium and an embryo, with 30 repetitions. The featured mediums were: MS (Murashige & Skoog, 1962), WPM – Woody Plant Medium (Lloyd & Mccown, 1980), QL (Quoirin & Leproivre, 1977) and N6 (Chu et al., 1975), supplemented with sucrose (30 $g.L^{-1}$), pH 5.8 and Phytagel® (2,2 $g.L^{-1}$).

Initially, the embryos were kept in the dark for seven days to minimize oxidation problems and were afterwards transferred to the growth room at 25 °C ± 5 °C and 16 photoperiod hours provided by 20 W fluorescent tubes kept at a distance of 25 cm from the tubes. At the 14-day *in vitro* mark the embryo's development was evaluated, using the formation of the primary root and complete opening of the cotyledons as criteria, for it grants autotrophic ability for the plant's establishment. Aerial part height (cm) and the number of expanded leaves were assessed on the 42nd day *in vitro*.

After this period, the plants were submitted to root wash and individually planted in 200 mL plastic cups filled with SUBRAS® commercial substrate humidified with 25 mL of water. Twelve plants were used per treatment. Coincident to the planting, in order to simulate wet chamber conditions, the 200 mL cups were covered with 400 mL plastic cups and the experimental units kept inside the growth room for pre-acclimatization for 15 days. Lastly, for acclimatization, the plants were transplanted to polyethylene bags (18 cm x 30 cm) with the

same substrate, and then kept in a tunnel greenhouse with 70% of shading. After 60 days, the survival (out of the total of acclimatized plants) was assessed, as well as the number of expanded leaves and stem diameter.

The data was submitted to a verification of outliers by the Grubbs test, error normality by the Shapiro-Wilk test and variance equality by the Bartlett's test. After the satisfaction of said assumptions, the analysis of variance was to follow, and when the F value implied difference between the treatments, their averages were compared by the Tukey's test.

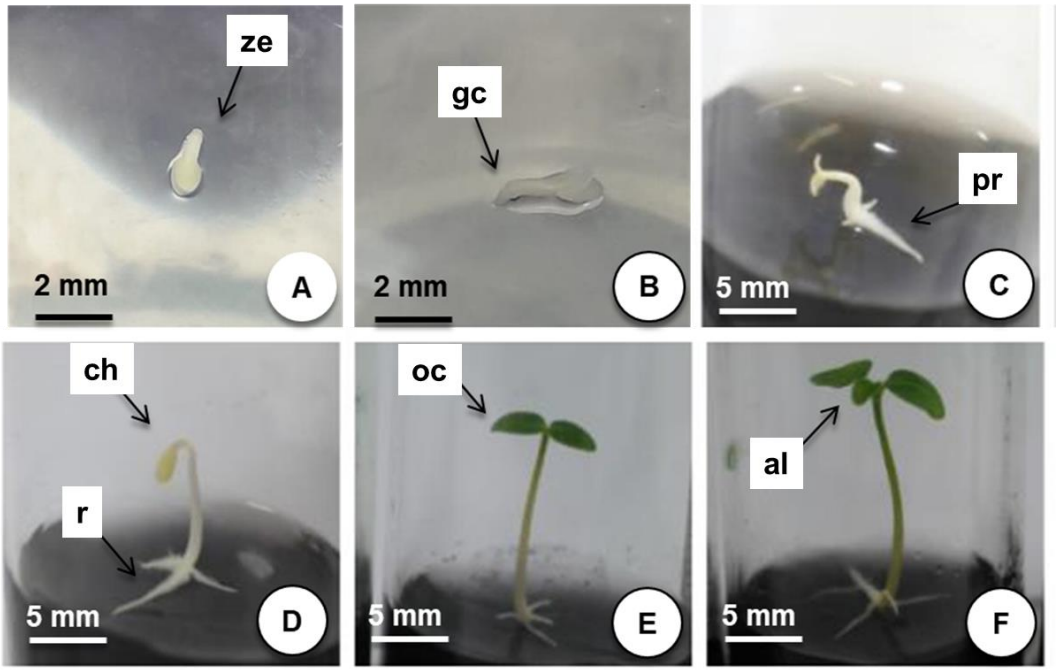
3. Results and Discussion

Embryo development and establishment of plants *in vitro*

Twenty-four hours after the start of *in vitro* cultivation, it was possible to observe the embryos' swelling and cotyledon opening initiation (Figure 1 A), which was followed by the hypocotyl-radicular axis' geotropic curvature (Figure 1 B) after forty-eight hours. After seventy-two hours, the primary root's development, presence of hairs and hypocolyte (Figure 1 C). On the seventh day of cultivation, under a 16-hour photoperiod, the progressive stretching of the hypocolyte, formation of the cotyledon hook and development of new roots could be verified (Figure 1 D).

After 14 days *in vitro* the total opening and production of pigments on the cotyledons, as well as the development of the primary root were observed (Figure 1 E), which characterize the criteria to tell an embryo is well developed. On the twenty-first day the emission of leaves from the epicotyl occurred (Figure 1 F), and on and in the following weeks there was epicotyl growth, expansion and formation of new leaves, besides greater development and establishment of the root system. Moreover, every embryo labeled as developed resulted in a normal plant, with a survival rate of 100% (data not shown).

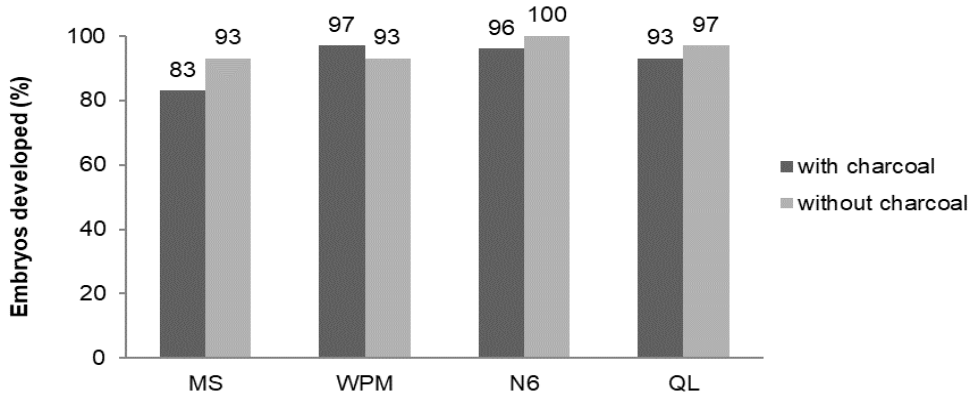
Figure 1 - Description of the observed events during the development of the *Croton lechleri* zygotic embryo *in vitro*. A – Swelling and early onset of cotyledons; B – Geotropic curvature onset. C – Root development. D – Hypocotyl elongation and cotyledonary hook formation. E and F – Developed embryo and 14 and 21-days old plant, respectively. Labels: ze - zygotic embryo; gc - geotropic curvature; pr - primary root; ch - cotyledonary hook; r – root; oc - open cotyledons; al - actual leaf



Source: Our authorship (2020).

Apart from the MS medium with active charcoal, in which 83% of the embryos developed, the remaining formulations (WPM, QL and N6) provided an average development over 90% (Figure 2).

Figure 2 - Percentage of developed Dragon's blood embryos as a function of the culture medium and supplementation with active charcoal, at 14 days of *in vitro* cultivation.



Source: Our authorship (2020).

The greatest difference associated to the addition of active charcoal was verified when the embryos were cultivated in the MS medium. Although the WPM and QL mediums contain 25% of the concentration of ammonium nitrate (NH_4NO_3) of the MS formulation (Oliveira et al., 2013), and being NH_4NO_3 absent in the N6 medium, no significant differences were observed in the percentage of developed embryos as a function of the culture medium's composition.

The medium's supplementation with active charcoal is conventional within protocols that utilize zygotic embryos as an explant. This substance may influence the *in vitro* morphogenesis, which depends on the culture medium's composition and chosen explant (Sanputawong et al., 2015). One of the consequences assigned to the presence of charcoal in the culture medium is the reduction of oxidation by phenolic compounds (Thomas, 2008) through the absorption of toxic substances released by the explant, and consequently promoting growth and development *in vitro* (Galdiano Júnior et al., 2012).

It was acknowledged a significant effect of the interaction between the factors (medium x charcoal). As seen on Table 1, for the number of expended leaves (NEL), only the N6 and WPM mediums differed ($p<0,05$) as to the presence or lack of active charcoal (AC).

Table 1 - Number of expanded leaves (NEL) and shoot height (SH) of the Dragon's blood plants cultivated in vitro in different culture medium formulations and supplementation with active charcoal, at 42 days.

Culture medium	NEL		SH (cm)	
	With charcoal	Without charcoal	With charcoal	Without charcoal
MS	2,48 bA	2,46 bA	3,59 bA	2,41 cB
N6	3,86 aA	1,37 cB	4,62 aA	3,20 bB
QL	3,64 aA	4,21 aA	3,11 bcA	3,35 abA
WPM	3,10 abB	4,03 aA	2,95 cB	3,86 aA
CV %	39,15		27,33	

Averages followed by the same letter, lowercase in the column (medium comparison) and uppercase in the line (presence or absence of charcoal), do not differ between each other by the Tukey test at 0,05 of probability. Source: Our authorship (2020).

The greatest NEL was verified when embryos were cultivated in N6 plus AC (3,86 leaves) and WPM devoid of charcoal (4,03 leaves). Without active charcoal the N6 medium featured the worst result for NEL (1,37), while the lowest number featured in the presence of AC occurred in the MS medium (2,48).

Regarding the height of the aerial part (HAP), the addition of active charcoal provided the highest and lowest averages in the N6 (4,62 cm) and WPM (2,95) mediums ($p<0,05$). An opposite result was verified in the absence of charcoal, with the greatest HAP on the WPM medium (3,86 cm), from which the QL medium (3,35 cm) did not differ, but was statistically superior to the MS (2,41 cm) and N6 (3,20 cm) formulations.

The highest HAP found for N6 supplemented with active charcoal was a result of a greater elongation of the hypocotyl. Plants produced in the WPM and QL culture mediums, with or without charcoal, had larger uniformity and more vigor. Sachs et al. (1959) state that the inhibition of cauline elongation may be attributed to the inhibition of either expansion or cell division. Calcium fulfills a basic action for said plant response, for an alteration in intracellular concentration may result in the inhibition of the aerial part growth, as well as in the elongation of cells and tissues (Aranda-Peres, et. al., 2009; Poothong & Reed, 2014).

For this present study, the largest concentrations of salts in the MS medium may have influenced the *in vitro* of the dragon's blood plants. The excess of nutrients can influence the nutritional balance for plant metabolism and cause losses on development. For macaw palm (Arecaceae) the greatest plant growth was observed when the zygotic embryos were cultivated in MS medium with 50% of saline concentrations (Soares et al., 2011).

Nitrogen may influence the *in vitro* growth and development due to its importance for the synthesis of amino acids and proteins (Sodek, 2004). The means (nitrate and ammonium) through which nitrogen is available in the culture medium are quickly assimilable and show primordial functions for cellular metabolism, such as maintenance of the intra and extracellular ionic balance (George et al., 2008). Besides, the minerals' influence on a dragon's blood growth and development may be related to the quantity of such substances in the medium, as well as their gathering in plant tissues.

Ex vitro survival and plant growth in acclimatization

Plants cultivated *in vitro* had 100% of survival rate in acclimatization, independent of the culture medium formulation and addition of active charcoal. It is worth noting that the pre-acclimatization stage was crucial for the survival and further adaptation of the plants in a greenhouse, with no presence of withering, yellowing or necrosis on the persistent leaves (which remained from the *in vitro* cultivation), which indicates adaptation of the plants with minimum environmental stress.

For plant growth, assessed at 60 days *ex vitro*, only the culture medium had a significative influence on the evaluated variables (Table 2), with the exception of the number of expanded leaves, which did not differ ($p<0,05$) between the chosen formulations and addition of active charcoal. Largest stem diameter (SD: 2,57 mm and 2, 45 mm) and shoot height (SH: 15,31 cm and 13,99 cm) were observed on plants deriving from QL and WPM mediums, which did not differ between each other ($p<0,05$). The lowest average for SD (1,92 mm) and SH (11,78 cm) occurred on plants cultivated *in vitro* in MS medium, followed by the N6 medium.

Table 2 - Stem diameter (SD) and shoot height (SH) of Dragon's blood plants acclimatized for 60 days, correlated to the originating medium used for *in vitro* cultivation of the zygotic embryo

Culture medium	SD (mm)	SH (cm)
MS	1,92 c	11,78 b
N6	2,25 b	12,81 b
QL	2,57 a	15,31 a
WPM	2,45 ab	13,99 ab
CV %	19,62	24,03

Averages followed by the same letter, in every variable, do not differ between each other by the Tukey test at 0,05 of probability. Source: Our authorship (2020).

The means through which nitrogen is provided and supplied to the plants influence on their growth and development (Pedroso-de-Moraes et al., 2012). The high concentrations of ammonium (NH_4^+) and nitrate (NO_3^-) in MS medium and the reduced concentration in N6, may have reduced the *in vitro* growth of the plants. According to Economou (2013) the reduction of NH_4^+ in MS medium improves the quality of woody plants in acclimatization. Turmeric plants cultivated *in vitro* in the absence of NH_4^+ and with high concentrations of calcium, had the largest growth compared to plants produced in MS medium with 100% of the concentration of ammonium (Adelberg et al., 2013). The reduction of cationic nitrogen in the MS medium also favored the growth in height during the acclimatization of turmeric plants (El-Hawaz et al., 2016).

4. Conclusion

With the conditions described in this study, it is suggested that the *in vitro* cultivation of *Croton lechleri* embryos be done in WPM or QL mediums, devoid of active charcoal. For the adaptation of the plants to the *ex vitro* environment it is recommended the use of a pre-acclimatization stage in a humid chamber system and growth room, for 15 days, followed by acclimatization in a tunnel greenhouse with 70% of shading.

As suggestions for future work, different types of ammonium and/or nitrate concentrations could be tested in culture mediums formulations.

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Percentage of contribution of each author in the manuscript

João Bosco de Oliveira Júnior – 20%

Camilla Mendes Pedroza Pessoa – 20%

Hendril da Silva Lopes – 20%

Frederico Henrique da Silva Costa – 20%

Jonny Everson Scherwinski Pereira – 20%