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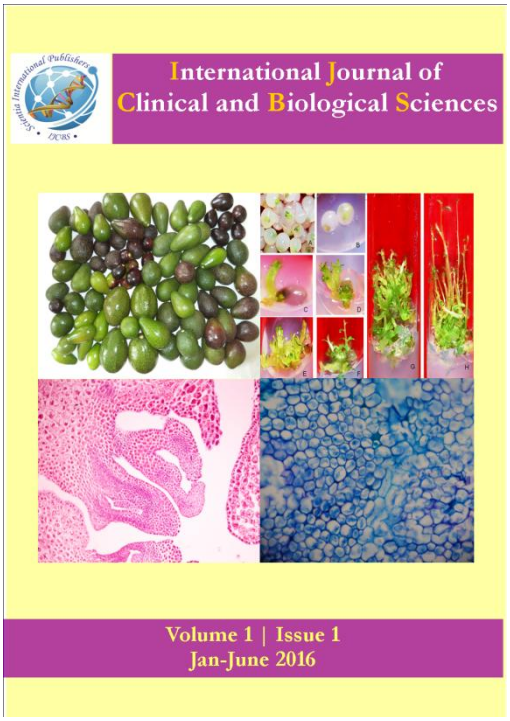

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Morphological and histological ontogeny of Somatic embryogenesis in Centrolobium ochroxylum

Quiroga S[*]*, Iles D, Morillo E, Orbe K, Meneses S, Buitrón J*
Autonomous National Institute of Agricultural Research (INIAP), Cutuglagua, Ecuador

Abstract

Centrolobium ochroxylum, a forest species is of great commercial value. Indirect somatic embryogenesis has been developed from leaf explants for *Centrolobium ochroxylum*. Seven different media were tested for the induction. Embryogenic callus was observed using half strength MS medium supplemented with different concentrations of picloram under complete dark conditions. Medium supplemented with 5 mg/l picloram showed the highest percentage (68%) of embryogenic callus. The histological observation revealed the presence of isodiametric cells with a prominent nucleus at the centre. Development of somatic embryos was also observed in another three media without plant growth regulators. Starch grains and storage proteins were observed in matured somatic embryos. This is the first report on somatic embryogenesis in *Centrolobium ochroxylum.*

Keywords: *Centrolobium ochroxylum*, somatic embryogenesis, picloram, darkness.

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Introduction

C. ochroxylum has been reported in the Red List of endemic species due to extensive wood chopping for furniture [1-5]. Conventional propagation of *C. ochroxylum* is limited due to low germination (36 to 42%) and rooting frequency (67%) from vegetative cuttings [1]. Thus, the use of tissue culture techniques such as somatic embryogenesis may become an important alternative to solve the above problems. Somatic embryogenesis results in regeneration of whole plant due to its totipotency nature [6, 7]. This *in vitro* culture technique is rapid and allow us to obtain a large number of clones from small tissues. In recent years, somatic embryogenesis has been reported in forest species related to the Fabaceae [8-10] as well as species of economic and nutritional importance [11-13]. However, *in vitro* studies of *C. ochroxylum* has not been reported so far. The main objective of the present study is to evaluate morphological and histological transition phases of somatic embryogenesis in *C. ochroxylum* from leaf explant and also to study the effect of light on somatic embryogenesis. This report is the first of its kind on somatic embryogenesis in *C. ochroxylum*.

[*]*Corresponding author: Dr. Quiroga S, Autonomous National Institute of Agricultural Research (INIAP), Cutuglagua, Ecuador, E-Mail: saquirogaq@gmail.com*

Materials and methods

Plant material

Young leaves were excised from 1-month-old *Centrolobium ochroxylum* plants grown at Estación Experimental Santa Catalina, INIAP, Cutuglagua, Ecuador. The leaves were first washed with detergent followed by water, then dipped in 0,3% Povidin® (active ingredient: 10% povidone-iodine) for 20 min, followed by antifungal solution (1% Carbendazim, 0.5% rifampicin and 1% Copper (II) sulfate pentahydrate) for 15 min. Later they were incubated for 24 hours at 4°C and then sterilized in 0.1% and 1% (v/v) sodium hypochlorite solution for 30 and 10 min respectively. Finally, the explants were washed four times with sterile distilled water. The leaves were cut into 15 x 15 mm and cultured with the abaxial surface in contact with the medium, except on M5 and M7 media with the adaxial surface.

Induction of embryogenic callus

Seven culture media were tested for induction of embyogenic callus: half-strength Murashige and Skoog (MS) medium with 40 g/l sucrose and 5 mg/l picloram (M1); half-strength MS medium with 40 g/ l sucrose, and 10 mg/l picloram (M2); B5 medium with 30 g/l sucrose,1 mg/l 2,4-D and 2 mg/l kinetin (M3); WPM medium with 30 g/l sucrose, 0.25 mg/l BAP, 2 mg/l ANA, and 1 g/l casein hydrolyzate (M4); MS medium with 30 g/l sucrose, 5 mg/l ANA and 5 mg/l picloram (M5); Gamborg major salts and MS minor salts with 30 g/l sucrose, 2.25 mg/l BAP, and 1.86 mg/l ANA (M6); MS medium with 30 g/l sucrose, 0.1 mg/l 2,4-D, and 0.01 mg/l TDZ (M7). All the culture media for induction and maintenance of various stages were maintained at pH 5.7 before adding 8 g/l agar and autoclaving at 121.5 °C for 20 min. Cultures were incubated at 28 ± 2°C under 16/8 h light (A1) and dark period (A2).

Culture maintenance

After 21 days of culturing, embryogenic calli were transferred on to M1, M2 half-strength MS medium supplemented with different concentrations of picloram (0.5 and 5 mg/l), 40 g/l sucrose and also on M6 medium for induction stage.

Embryo formation, maturation, and germination

Nine culture media were tested after 23 days of culture on induction media, MS medium with 30 g/l sucrose, 8 g/l agar (F1); half-strength MS medium with 30 g/l sucrose, 0.5 g/l activated charcoal, and 8 g/l agar (F2); MS medium with 40 g/l sucrose, 30 g/l sorbitol, and 8 g/l agar (F3); half-strength MS medium with 50 g/l sucrose, 50 ml/l coconut water, 100 mg/l myo-inositol, and 8 g/l agar (F4); MS medium with 30 g/l sucrose, 2 mg/l ABA, and 3 g/l gelrite (F5); half-strength MS medium with 30 g/l sucrose, 500 mg/l glutamine, 200 mg/l casein hydrolysate, 0.1 mg/l ABA, and 8g/l agar (F6); half-strength MS medium with 30 g/l sucrose, 40 mg/l glutamine, 20 ml/l coconut water, and 8 g/l agar (F7); MS medium with 45 g/l sucrose, 20 ml/l coconut water,

and 8 g/l agar (F8); MS medium with 45 g/l sucrose, 20 ml/l coconut water, 1 g/l activated charcoal, and 100 mg/l PVP (F9). Cultures were incubated at 28 ± 2°C under 16/8 h light (A1) and dark period (A2). After 30 days of culture somatic embryos germinated on half-strength MS medium with 30 g/l sucrose, 10 ml/l coconut water, 1 mg/l AG3, and 7 g/l agar.

Histology studies

Embryogenic calli and somatic embryos were fixed in FAA (formalin: acetic acid: 70% alcohol in 1:1:18) solution, dehydrated in an ethanol series and embedded in paraffin wax. Longitudinal sections were cut at 10 µm and finally samples were stained with toluidine blue for 5 min [14].

Statistical analysis

Petri dishes with 5 explants were used per treatment for induction and maintenance stages, the experiment was repeated five times and the Kruskal-Wallis non-parametric test was applied. For maturation stage glass bottles (100 mm x 60 mm) with 4 embryogenic calli were used per treatment, the experiment was repeated three times and statistical hypothesis testing was applied.

Results and discussion

Induction and maintenance of embryogenic callus

Growth regulators in M3, M4, M6, and M7 media showed high percentage of callus formation (>96%); however, these media were ineffective for the formation of pro-embryogenic masses (PEMs). Embryogenic callus formation was observed only in three treatments (M1A1, M5A2, and M2A2). The Kruskal-Wallis non-parametric test showed statistically significant differences in these treatments during induction stage from day 14 ($p<0.0001$).

Table1: Percentage of embryogenic callus cultivated on seven induction culture media (M1-M7) under 16/8 h light (A1) and dark period (A2) for 7, 14, 21, 28 and 35 days

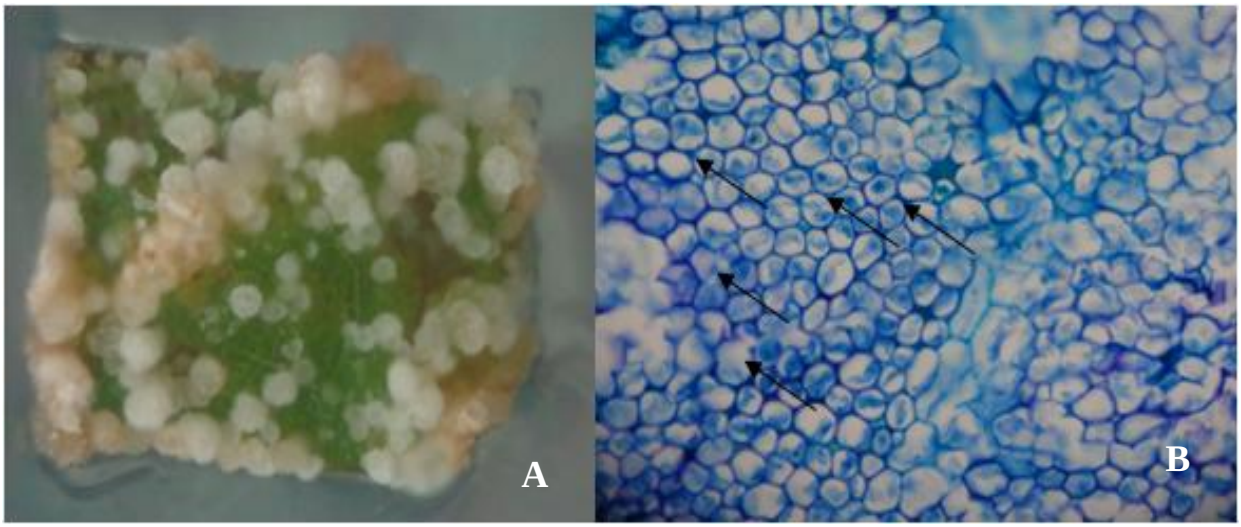
	Percentage of embryogenic callus (%)						
	M1	M2	M3	M4	M5	M6	M7
A1-14 days	0^a	0^a	0^a	0^a	0^a	0^a	0^a
A2-14 days	28^b	24^b	0^a	0^a	20^b	0^a	0^a
A1-21 days	0^a	0^a	0^a	0^a	0^a	0^a	0^a
A2-21 days	68^b	56^b	0^a	0^a	48^b	0^a	0^a
A1-28 days	0^a	0^a	0^a	0^a	0^a	0^a	0^a
A2-28 days	68^b	56^b	0^a	0^a	48^b	0^a	0^a
A1-35 days	0^a	0^a	0^a	0^a	0^a	0^a	0^a
A2-35 days	44^b	36^b	0^a	0^a	28^b	0^a	0^a

Values with the same letter are not significantly different at the 0.05 level of confidence

M1A2 (half-strength MS medium with 40 g/l sucrose, 5 mg/l picloram and 8 g/l agar, under complete dark) media had the highest percentage of embryogenic callus, showing values of 28%, 68%, and 44% at 14, 21, and 35 days respectively without statistical differences with respect to M2A2 (half-strength MS medium with 40 g/ l sucrose, 10 mg/l picloram and 8 g/l agar, under complete dark) and M5A2 (MS medium with 30 g/l sucrose, 5 mg/l ANA, 5 mg/l picloram and 8 g/l agar, under complete dark) media. These three media contained picloram in their formulation; it was the key auxin involved in cell reprogramming, activation of embryogenic potential and formation of proembriogenic masses (PEMs) in *C. ochroxylum* [15-17].

Histological analysis of calluses (Figure 1) from M1, M2 and M5 media under dark adapted conditions (A2) showed small cells with prominent isodiametric nucleus and characteristics of embryogenic cells [18]. Calluses were observed with hyperhydration from M1 to M7 media under a 16/8 h light (A1), showed broken cells in all treatments with a clear light sensitivity, causing a change in morphology and cell structure. While, large cells were observed with no defined nucleus (non-embryogenic cells) in the same culture (only M3, M4, M6, M7 media) under continuous darkness [8, 19, 20].

Figure 1: Embryogenic callus (EC) in *Centrolobium ochroxylum*. A) EC from M1A2 treatment B) Histological analysis of EC (40x)



During maintenance phase, all cultures showed a decrease in the percentage of embryogenic callus. At 21 days of culturing, no embryogenic callus was observed; this may be due to continuous presence of auxin in culture medium which results in cell elongation and cell alteration, reducing embryogenic competence of cells [21].

Embryo formation, maturation, and germination

After 23 days of culturing on the induction medium, embryogenic calluses were transferred to nine media lacking plant growth regulators (PGRs). A total of 18 somatic embryos were obtained in three culture media after 30 days of culturing (Table 3). Nine viable embryos were observed, while nine somatic embryos showed changes in their morphology. Removal of PGRs from culture medium, results in stop of cell division, consequently formation of somatic embryos in globular state started [21]. It has also been described that the genes involved in blocking of the transition to heart stage are removed due to the suppression of auxin [22, 23], affirmation that probably occurred in *C. ochroxylum* during somatic embryo expression.

At this stage a statistical hypothesis testing was performed for a parameter at $\alpha = 0.05$. The null hypothesis was rejected $Z_0 > VC$ (3.50 > 1.96) therefore statistically we inferred that there is the capacity for expression of somatic embryos from embryogenic callus of *C. ochroxylum.*

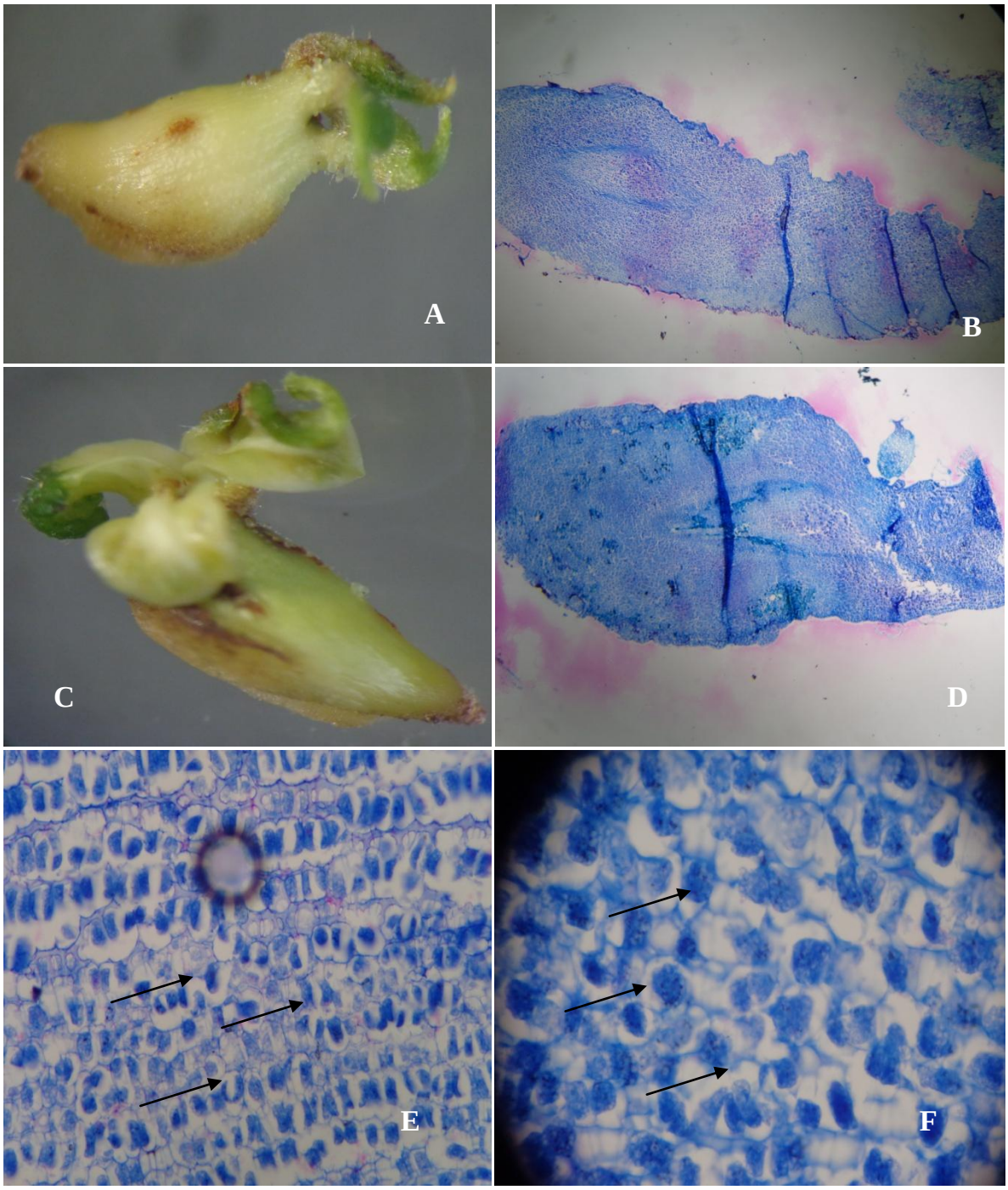
Table 2: Total somatic embryos during embryo formation and maturation stage cultivated on nine culture media (F1-F9) under 16/8 h light (A1) and dark period (A2) after 30 days of culturing

	Total somatic embryos								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
A1	0	5	0	0	0	0	0	0	6
A2	0	0	0	0	0	0	0	5	2

The maturation process during somatic embryogenesis is characterized by the accumulation of storage substances. It has been described that coconut water plays an important role in the accumulation of these substances (Figure 2), assertion observed in this study with a concentration of 20% v/v of coconut water [24-26].

The use of antioxidants as activated charcoal or PVP appear to exert a positive influence on *C. ochroxylum* during maturation stage due to the reduction of the effects caused by the formation of quinones and oxidation of polyphenols, compounds highly reactive and toxic for tissues that cause a profound physiological effect during somatic embryogenesis [8, 27]. Activated charcoal and PVP were effective in embryo formation and maturation of somatic embryos. Somatic embryos were observed in F2, F8, and F9 media containing these compounds. Moreover, somatic embryo formation is resisted by stress in *C. ochroxylum*.

Figure 2: Histological analysis of mature somatic embryos; (A) torpedo stage, (C) Cotiledonar stage. (B, D): Histological analysis of somatic embryos shown in (A) and (C) (4x); (E, F): Histology of mature somatic embryo, the arrows point to the accumulation of storage substances (40x)



In the present study, we have observed germination of normal and abnormal embryos on a half-strength MS medium supplemented with 30 g/l sucrose, 10 ml/l coconut water, 1 mg/l AG3 and 7 g/l agar (Figure 3). Genotype also plays an important role in the germination of somatic embryos [8,28,29]. It was not possible to establish a reliable germination method in *C. ochroxylum* due to low number of somatic embryos involved at this stage. However, it is the first report in *C. ochroxylum* and this result was included for this species due to the complexity involved in the process of somatic embryogenesis.

Figure 3: Germination of somatic embryos in *C. ochroxylum*

Conclusion

Dark and picloram played an essential role in *C. ochroxylum* induction. Only picloram under continuous darkness showed pro-embryogenic masses. The highest expression of embryogenic potential was observed after 21 days of induction, histological analysis allowed to observe cells with prominent isodiametric nucleus. The proliferation and maintenance stage was not viable due to the negative effect of PGRs in the culture media. The expression of somatic embryos was obtained without growth regulators and supplemented with antioxidants as activated charcoal or PVP, determining the capability of *C. ochroxylum* to express somatic embryos from embryogenic callus (Z_0 = 3.50). Histological observation of mature somatic embryos showed the accumulation of storage substances. Only two somatic embryos germinated however, these results were inconclusive.

Conflict of Interest

We declare that we have no conflict of interest

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