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Establishing a Callus Culture of *Cnidoscolus chayamansa* McVaugh: A Species with Ethnopharmacological Value

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Abstract

Cnidoscolus chayamansa is a plant widely used as food with potential medicinal applications. Plant tissue culture enables the production of secondary metabolites from plants in a sustainable manner. The aim of this work was to establish a callus culture of *C. chayamansa* using leaf explants, which, in the future, can be used to obtain whole extracts or specific compounds of interest. Different concentrations and combinations of *6*-B*enzylaminopurine* (BAP) and 2,4-Dichlorophenoxyacetic Acid (2,4-D) in Murashige and Skoog (MS) culture medium were evaluated for their ability to support callus induction from immature leaf explants. Highest callus yield (100%) was achieved when leaf explants were incubated in MS medium supplemented with BAP (5.0 mg L^{-1}) and 2,4-D (2.5 or 5.0 mg mL^{-1}). A reduced rate of callus induction (75%) was observed when leaf explants were exposed to lower concentrations (0.5 mg L^{-1}) of BAP or 2,4-D. The calluses obtained in this work can be used as a biotechnological alternative for *in vitro* propagation of *C. chayamansa* and for obtaining extracts or compounds with a possible pharmacological application.

Keywords: Chaya, *In vitro* culture, Medicinal plants, Plant growth regulators.

Introduction

Cnidoscolus chayamansa, commonly known as "tree spinach" or "chaya" in Central and South America, is a semi-perennial and semi-woody shrub. *C. chayamansa* is an important staple leafy vegetable for the autochthonous people of Mesoamerica. The nutritional value of *C. chayamansa* [1] and traditional medicinal and food use has been established in the literature [1,2]. As a food for freshwater fish, partial substitution of balanced feed with this leaf has been determined to be viable for tilapia (*Oreochromis niloticus* L.) culture in the integrated agricultural–aquaculture systems in Yucatan, Mexico [3]. The antioxidant capacity and phenolic content of the leaf extracts, in addition to anti-inflammatory and cardioprotective properties, has been extensively described in the literature [4-6]. Furthermore, the antioxidant and antimutagenic activity of a methanolic extract from the edible leaves of *C. chayamansa* has been detailed, in addition to its antidiabetic effects in diabetic rats [7,8]. A phytochemical profile, including flavonoids, phenolic acids, saponins, and alkaloids, and the antioxidant properties of fresh leaves were recently reported [8]. Antimycobacterial (MIC $\leq$ 50 µg mL^{-1}), Antibacterial (*P. aeruginosa* and *E. coli*), Antiprotozoal ($IC_{50} \leq 50$ µg mL^{-1}), and Anti-Inflammatory (ED_{50} = 1.66 mg ear^{-1} for the TPA model and 467.73 mg kg^{-1} for the carragenine model) activities of the leaf extract ($CHCl_3$: MeOH, 1: 1) have been published. Also, kaempferol-3,7-dimethyl ether, 5-hydroxy-7-3',4'-

trimethoxyflavanone, moretenol, and, 'moretenol acetate were isolated and characterized. Toxic acute and subacute effects (LD_{50} of extract > 2 g kg^{-1}) were estimated in a sub-acute toxicity test using diabetic rats; the extract was also administered at 1 g kg^{-1} for 28 days and did not cause lethality or any alteration in hematological and biochemical values; in addition, histological analysis of the liver, kidney, and spleen revealed no structural changes [9].

As mentioned, several works have reported on the properties and benefits of *C. chayamansa*; however, biotechnological tools have not been used to exploit this species in a sustainable manner. In these regard, plant cell culture represents an advantageous system for the study of different cellular processes, since the conditions can be strictly controlled, thereby allowing the effects of a single factor on a given process to be monitored. [10] In addition, this technique could serve as an invaluable tool for agricultural and pharmaceutical companies around the world to propagate these plants and produce secondary metabolites [11]. Toward this end, callus induction is the necessary first step, as in many tissue culture experiments, including cell suspension culture or indirect organogenesis [12]. According to the literature, a callus culture of *C. chayamansa* has not yet been reported. The aims of this study were to evaluate the influence of different combinations and concentrations of Plant Growth Regulators (PGRs) on callus induction in *C. chayamansa* leaf explants and to monitor the phenotypic characteristics of the callus culture, such as color, friability, and proliferation rate.

Materials and Methods

Plant Material

Cnidoscolus chayamansa (Mc Vaugh) was collected in Mexico City, Mexico, in June 2016. The plant was identified by M.Sc. Abigail Aguilar of the Instituto Mexicano del Seguro Social (IMSS) and a voucher specimen (16252) was deposited at the IMSSM Herbarium.

Aseptic Conditions

Immature leaf explants of 1-2 cm in length were excised from the plant, washed with a soap solution for 10 minutes, and rinsed with running tap water. Then, leaf explants were immersed into an aqueous solution with Ampicillin (100 mg mL^{-1}), Tetracycline (200 mg mL^{-1}), and Cefotaxime (15 mg mL^{-1}) for 30 min; followed by another immersion in an aqueous mixture with Shogum® (0.01 mL mL^{-1}) and Fungoxyl® (0.01 g mL^{-1}) for 20 min. Later, explants were immersed into a 70% (v/v) ethanol for 45 s; 0.9% (v/v) sodium hypochlorite with Tween-20 (three drops per 100 mL of solution) at low shaking for 20 min. Afterwards, under aseptic conditions, leaf explants were rinsed three times in sterile deionized water, followed by rinsing and segmenting in a sterile antioxidant solution (100 mg citric acid and 150 ascorbic acid mg L^{-1}) for 15 min.

Culture Medium and Incubation Conditions

The basal culture medium consisted of Murashige and Skoog (MS) [12], supplemented with 3% (w/v) sucrose, 100 mg L^{-1} citric acid, and 150 mg L^{-1} ascorbic acid. For callus induction from immature leaf explants, the culture medium was supplemented with different combinations and concentrations of the cytokinin *6-Benzylaminopurine* (BAP) and the auxin 2,4-Dichlorophenoxyacetic Acid (2,4-D). Both PGRs were used in concentrations of 0.00, 0.5, 1.0, 2.5 and 5.0 mg L^{-1} at pH 5.8. Phytagel (0.2% w/v) was used for solidifying the culture medium. 20 mL of culture medium were poured into culture tubes (150 X 20 mm). Culture medium were sterilized in an autoclave at 121°C and 15 psi for 18 minutes. Cultures were incubated at 26 ± 2°C under a photoperiod with 16 hours of light using white florescent lighting (50 µmol m^{-2} s^{-1}). Eight tubes with an explant were used to evaluate callus induction for each treatment. The explants exhibiting a callus or morphogenic responses were expressed as a percentage of the total explants, which was determined after 30 days of culture. The treatments that induced the highest percentages were sub-cultured every 30 days on fresh media.

Results

Although immature leaves were used for callus induction, problems with explant contamination and oxidation were observed, which drastically hindered their growth. To resolve these problems, an antioxidant solution (100 mg citric acid and 150 mg ascorbic acid L^{-1}) was added to the culture medium. Furthermore, this antioxidant solution was used during the rinsing and cutting of the explants. An antifungal solution containing Shogún® 0.01 mL mL^{-1} and Fungoxyl® 0.01 g mL^{-1} was also used during the disinfection process. These treatments reduced the stress and contamination caused by wounding and unwanted microorganisms and, thereby, enhanced explant growth.

Control treatment (CDB0) and the treatments from CDB1 to CDB5, CDB7, and CDB9 to CDB22 were not able to produce callus or induce morphogenic responses. Treatments CDB6 (0.5 mg L^{-1} BAP with 0.5 mg L^{-1} 2,4-D) and CDB8 (0.5 mg L^{-1} BAP with 2.5 mg L^{-1} 2,4-D) yielded 75% callus induction with a compact greenish appearance for B6 and a compact brown appearance for B8. Highest callus induction (100%) was achieved when leaf explants were supplemented with CDB23 (5.0 mg L^{-1} BAP and 2.5 mg L^{-1} 2,4-D) or CDB24 (5.0 mg L^{-1} BAP and 5.0 mg mL^{-1} 2,4-D) (Table 1). The CDB23 and CDB24 treatments produced friable greenish callus (Figure 1).

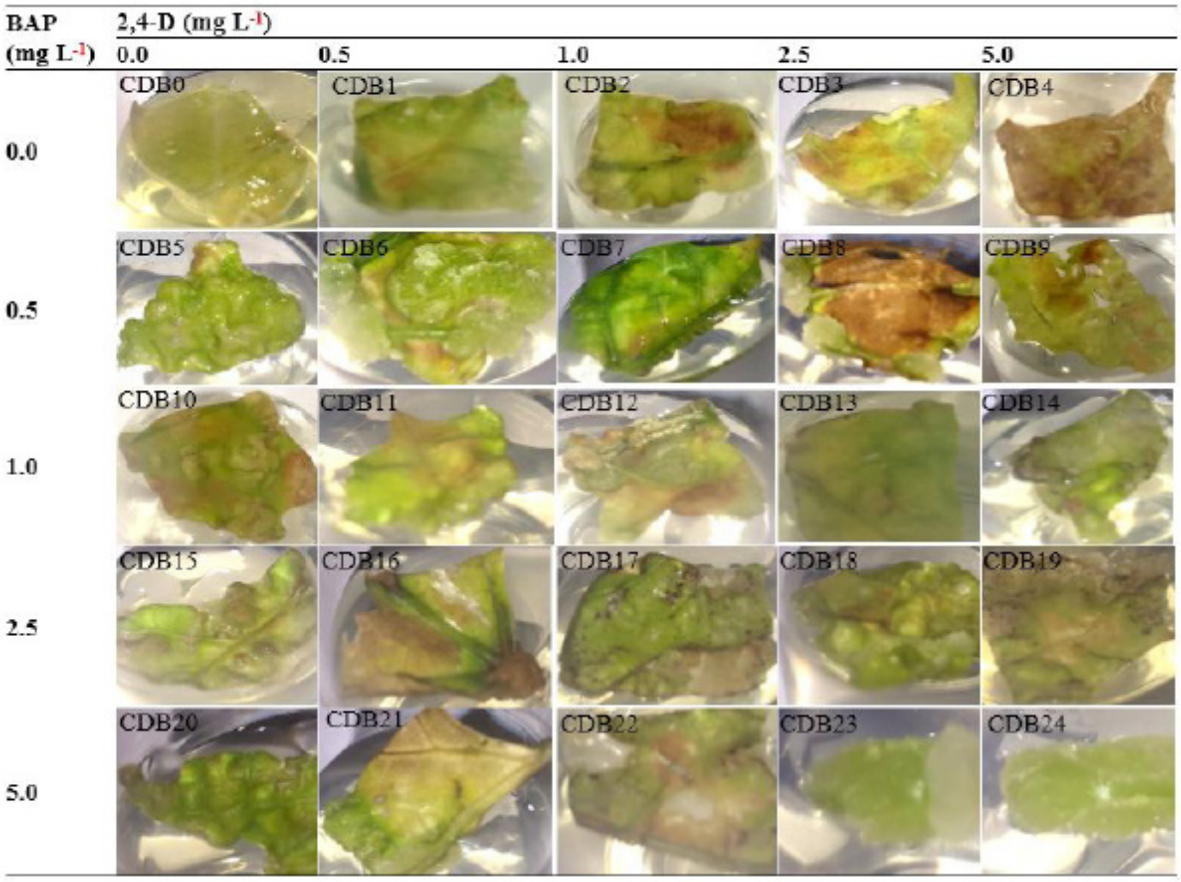

Figure 1: Effect of BAP and 2,4-D on leaf explants of *C. chayamansa* after 30 days of culture.

Treatment code	PGRs (mg L^{-1})		Callus induction (%)	Appearance of the callus
	BAP	2,4-D		
CDB0	0	0	0	–
CDB1	0	0.5	0	–
CDB2	0	1	0	–
CDB3	0	2.5	0	–
CDB4	0	5	0	–
CDB5	0.5	0	0	–
CDB6	0.5	0.5	75	compact greenish callus
CDB7	0.5	1	0	
CDB8	0.5	2.5	75	compact brown callus
CDB9	0.5	5	0	–
CDB10	1	0	0	–
CDB11	1	0.5	0	–

CDB12	1	1	0	–
CDB13	1	2.5	0	–
CDB14	1	5	0	–
CDB15	0	2.5	0	–
CDB16	0.5	2.5	0	–
CDB17	1	2.5	0	–
CDB18	2.5	2.5	0	–
CDB19	5	2.5	0	–
CDB20	5	0	0	–
CDB21	5	0.5	0	–
CDB22	5	1	0	–
CDB23	5	2.5	100	friable greenish callus
CDB24	5	5	100	friable greenish callus
PGRs: Plant Growth Regulators; BAP: 6-Benzylaminopurine (BAP); 2,4-D: 2,4-Dichlorophenoxyacetic Acid.				

Table 1: Callus induction (%) on immature explants from *C. chayamansa* leaves under different concentrations and combinations of BAP and 2,4-D, after 30 days of culture.

Discussion

To the best of our knowledge, this is the first study to establish a callus culture from the *C. chayamansa* leaf. Similar studies have described callus induction with BAP and 2,4-D on hypocotyl, stem, node, and leaf explants of *Lopezia racemosa*, in which, the leaf explants showed the highest callus formation with 0.5 mg L^{-1} BAP along with 1.0 mg L^{-1} 2,4-D; from this, the 6-O-palmitoyl-3-O-β-D-Glucopyranosylcampesterol compound was isolated, which exhibited anti-inflammatory activity [13]. Other similar species from the Euphorbiaceae family, such as *Manihot esculenta*, produced callus at 100% efficiency when shoot apical meristems with one to two leaf primordia were cultured on MS medium supplemented with 0.1 mg L^{-1} BAP, 0.2 mg L^{-1} Naphthaleneacetic Acid (NAA), and 0.25 mg L^{-1} *Gibberellic Acid* (GA_3) [14]. *Phyllanthus stipulatus* showed callus formation and growth when nodal segments of explants were cultured under light conditions on MS medium supplemented with 5.0 mM NAA or under dark conditions in MS medium supplemented with 5.0 mM NAA or BAP or N^6-(2-isopentenyl) adenine (2iP) (1.25 - 5.0 mM) [15].

Conclusion

Different concentrations and combinations of BAP and 2.4-D in MS medium were evaluated in immature leaf explants of *C. chayamansa* to establish callus cultures. In future studies, this callus can be used to initiate cell suspension cultures and obtain extracts and pure compounds.

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