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Research Article
Establishment of Callus and Cell Suspensions Cultures of *Dalbergia congestiflora* (Fabaceae) to (+)-Medicarpin Production

[1]A. Hernández-García, [1]C. Velázquez-Becerra, [1]R. Herrera-Bucio, [2]J.J. García-Magaña, [1]P. López-Albarrán and [1]E. Ambriz

[1]Universidad Michoacana de San Nicolás de Hidalgo, Ave. Francisco. J. Múgica S/N, C.P. 58030, Morelia, Michoacán México
[2]Universidad Michoacana de San Nicolás de Hidalgo, Paseo de la Revolución No. 1, Colonia E. Zapata, C.P. 60180, Uruapan, Michoacán México

Abstract

Background and Objective: *Dalbergia congestiflora* Pittier heartwood extracts have shown cytotoxic and antifungal activity. The medicarpin obtained of heartwood has antifungal and cytotoxic response against some diseases. This study aimed to establish the callus and cell suspensions cultures of *D. congestiflora*, as well as the extraction and identification of medicarpin. **Materials and Methods:** Leaf explants from *in vitro* shoots were cultured on Murashige and Skoog medium (MS) supplemented with different concentrations of benzylaminopurine (BAP) and Naphthalene Acetic Acid (NAA) to induce callus formation. The cell suspensions were established on liquid medium optimum to callus growth, inoculating 0.5 g fresh weight and agitated on orbital shaker with subcultures each 16 days. Fresh, dry weight and (+)-Medicarpin were determined at 30 days of culture in callus and every four days until day 24 in cell suspensions. (+)-Medicarpin obtained from callus and cell suspension was identifying and quantified by ^{1}HNMR mass spectroscopy analyses. **Results:** The optimum media to callus induction was obtained with the combination of 1.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA and was used to establish the cell suspension cultures. The growth kinetics revealed a maximum cell grows that day 16 and 80% cell viability. Both callus and cell suspensions produced (+)-Medicarpin, obtaining 3.56 mg (+)-Medicarpin/g FW at day 14 in cell suspensions. **Conclusion:** The results of this study demonstrate the viability to establish the callus and cell suspension cultures of *D. congestiflora*. The both types of cells produce (+)-Medicarpin, which is the same compound founded in heartwood of *D. congestiflora*.

Key words: *Dalbergia congestiflora*, callus, cell suspensions, (+)-Medicarpin, phytoalexin

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Corresponding Author: E. Ambriz, Universidad Michoacana de San Nicolás de Hidalgo, Ave. Francisco. J. Múgica S/N, C.P. 58030, Morelia, Michoacán México

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Dalbergia congestiflora Pittier is a tree species commonly known as campinceran that it has showed cytotoxic activity against different diseaseas[1].Its heartwood is considered to have great beauty and is highly valued in the international market due to its excellent physical and mechanical properties for its use in the furniture industry and it's resistant to fungal decay[2]. Additionally, this tree is a recognised source of interesting substances such as pigments (neocandenatone)[3] or the pterocarpan phytoalexin medicarpin[4]. The neocandenatone content of the wood was measured by Barragán *et al*.[5] with approximately 4.85% of its dry weight, meanwhile the potential use of medicarpin is diverse because it has antifungal activity[2,4,6], sensitizes myeloid leukaemia cells to TRAIL-induced apoptosis and present osteogenic properties[7]. However, to take advantage from those achievements, authors required large amounts of heartwood, which involves cutting down several trees of this endangered species[8]. Concerning medicarpin, it is necessary to be able to produce it through cell cultures instead of the heartwood of this endangered tree.

Although cell suspensions were developed since the last century for the production of secondary metabolites of various plants, there are few studies for the establishment of cell suspensions for trees[9]. Naturally, the main advantage of cell suspension culture of campinceran is the possibility to obtain its secondary metabolites, particularly (+)-Medicarpin, regardless of the climate, season, weather, soil conditions, optimization and standardization of growing conditions. Obviously, the culture of plant cells, tissues and organs is an alternative way to produce appropriate secondary metabolites[10,11].

Optimal callus development of several tree species has been achieved in media with Naphthalene Acetic Acid (NAA) and kinetin for *Taxus*x *media* Rehd.[12], NAA and benzylaminopurine (BAP) for *Eucalyptus cinerea*[13] and NAA and BAP for *Dalbergia sissoo* Roxb.[14]. Those studies report that callus of tree species has been used to obtain secondary metabolites like taxol, anthraquinones or thymol[13,14].

The production of medicarpin in cell suspensions has only been studied in *Medicago sativa*[15] and *Cicer arietinum*[16,17], where the addition of elicitors is necessary to achieve optimal levels of this compound. However, these studies did not report whether they obtained a single conformer for medicarpin or its racemic mixture. Only the (+)-Medicarpin has been identified in the hexane extract from heartwood of campincerán[4], thus it was considered that cell cultures of *D. congestiflora* can be a reliable source of (+)-Medicarpin. The objective of this work was to develop an efficient protocol to establish callus and cell suspensions of *D. congestiflora* and the extraction and identification of the medicarpin.

MATERIALS AND METHODS

Plant material: The study was carried out from September, 2017 to December, 2019 (28 months) at Plant Biotechnology Laboratory of the Institute of Chemical and Biological Research of the Michoacana University of San Nicolás de Hidalgo. *In vitro* shoots of *Dalbergia congestiflora* Pittier from stem cuttings adult plants cultured in greenhouse conditions (Agro system)[18], were established. The explants were surface sterilized following the same methodology used by Cortés-Rodríguez *et al*.[19]. In aseptic conditions, sterilized explants were rinsed three times in sterile distilled water and leaves and basal portions of shoot tip were removed. Explants of 0.5 cm in length with apical buds were cultured in 125 mL baby food jars (polypropylene-covered) with 20 mL of Murashige and Skoog medium (MS)[20] with agar (8 g L^{-1}), sucrose (30 g L^{-1}), pH of 5.7 and 0.05 mg L^{-1} BAP to induce shoot regeneration. After 30 days of culture, single shoots per explant (5 cm long with three pair leaves) were used as leaf explants source to callus induction.

All cultures were maintained in a culture room at 25±1°C, with a 16 hrs photoperiod (26 µmol m^{-2} seg). Chemical and reagents used in this study were from J. Baker and Sigma Aldrich (Mexico).

Callus induction: Leaf explants (1.0 cm^2) were cultured on MS supplemented with different concentrations of BAP (0, 0.1, 0.25, 0.5, 1 and 2 mg L^{-1}) in combination with NAA (0 and 0.1 mg L^{-1}). At 30 days of culture, Fresh Weight (FW) and Dry Weight (DW) were evaluated to choose medium with higher production of callus. Fresh weight was directly determined by placing each portion of callus on a previously weighted filter paper and for estimating DW, the samples were dried at 50°C for 24 hrs and weighed in an analytical balance (MC1, Analytic AC Sartorius, USA). Friable callus was regularly subculture on fresh media by four-week intervals.

Cell suspension culture: Cell suspension cultures were established by transferring 2.5 g FW of 30-day old friable callus into 250 mL Erlenmeyer flasks containing 50 mL of fresh liquid media (MS with optimal growth callus) lacking agar. The suspension cultures were subculture in the MS liquid medium at 16 days-intervals agitated on a rotary shaker (120 rpm) and kept in a culture room conditions.

Cell suspension growth kinetics: The growth kinetics of *D. congestiflora* cell suspension were performed, preparing a cell suspension culture stocks to obtain 0.5 g mL^{-1} FW from 16 days-old cell suspensions with three subcultures, adding 1 mL into 10 mL of fresh medium MST10 in 50 mL Erlenmeyer flasks and cultured by 24 days. The suspension cultures were shaken at 120 rpm.

For determination of cell suspension growth curve, the FW (g) and DW (g) were recorded every four days until day 24. Cells were separated from the medium by filtration using previously weighted filter paper, applying vacuum to filtrate for one min and weighed as fresh weight. To obtain DW, the cells on filter paper were dried at 50°C for 24 hrs and then weighed in the analytical balance.

Cell viability: Cell viability was determined every four days by Widholm method[21], employing fluorescein diacetate (FDA) stored in an acetone stock solution (5 mg mL^{-1}) at 0°C. The staining was carried out mixing 10 µL of the FDA solution with 10 µL of the cell suspension. After 1 min at room temperature, the cells were examined for fluorescence using a fluorescent microscope (Nikon, model 14691, Japan). The viability was expressed as a percentage (%) and it is the relation between number of fluorescent cells and total number of cells.

Purification, identification and quantification of medicarpin: A 10 g FW of callus and cell suspensions of *D. congestiflora* were collected during exponential phase, pulverizing in liquid nitrogen and then subjected to extraction by cold maceration in 100 mL of dichloromethane. The extracts were filtered and evaporated to dryness on a vacuum rotary evaporator at 35°C, suspended in 1 mL of dichloromethane and stored at -20°C until analysis. Medicarpin accumulation both callus as cell suspensions systems was purified by Thin Layer Chromatography (TLC) and their identification was made by ^{1}HNMR mass spectroscopy. Data were recorded on a Varian Mercury-400BB spectrometer (400 MHz for ^{1}H and 100 MHz for 13C NMR) in $CDCl_3$ with TMS as internal standard.

(+)-Medicarpin accumulation in callus and cell suspension: Cell suspension of 10 mL of liquid medium were sampled every four days during growth kinetics and filtered through Whatman paper No. 1 in vacuum during 1 min. The 0.5 g FW was homogenized to medicarpin extraction and quantification for ^{1}HNMR mass spectroscopy. The accumulation of (+)-Medicarpin was expressed as mg g^{-1} FW.

Statistical analysis: A statistical analysis of FW, DW and (+)-Medicarpin production of cell suspension was carried out through one-way ANOVA and Tukey tests (n = 3) in the JP8 statistical package.

RESULTS

The effects of different concentrations of BAP and NAA were tested for induction and proliferation of callus from *D. congestiflora* leaf explants. After eight days of culture, all concentrations of BAP and NAA presented callus formation in 100% of *D. congestiflora* leaf explants, although the treatment 10 (MST10), with 1.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA carried the best growth of *D. congestiflora* callus (2.3 g FW and 0.8 g DW) and the best quality with whitish and friable callus at 30 days of culture. This treatment was significantly higher than all other investigated. A concentration of 1 mg L^{-1} BAP with 0 mg L^{-1} NAA was the second-best treatment for callus growth (Table 1).

Cell suspensions: The MST10 medium was used to establish cell suspension culture of *D. congestiflora*, obtaining cell division, a few cell aggregates and free cells some of which had a spherical form. At 16 days of the first culture, the cells showed 80% viability, a value that was maintained in the following three subcultures and during growth kinetic. Cell suspensions were subcultured every 16 days in fresh MST10 medium.

With the growth kinetics it was shown than cell suspensions started an exponential growth phase at day 4 that lasted for eight more days, after a short adaptation period during the first four days of subculture. The fresh weight had its greatest increase from day 8 to day 12 and remained stable from 16-24 days (Fig. 1a). While dry weight had its greatest increase immediately after than adaptation period, this is from 4-8 days and the higher value (0.1 g per 10 mL DW) was observed at day 16 (Fig. 1b). The cell degeneration and necrosis became apparent from day 24.

Identification of medicarpin: The mass spectra of compound purified by TLC of cell extracts from callus and cell suspensions of *D. congestiflora* showed a molecular ion at m/z = 270.0892 equivalent to the molecular formula $C_{16}H_{14}O_4$ (Fig. 2), which corresponds to (+)-Medicarpin compound. From the ^{1}HNMR analysis were found the following characteristics: HNMR δ 7.37 (d, J = 8.4 Hz, 1H, H-1), 6.55 (dd, J = 8.4, 2.6 Hz, 1H, H-2), 6.42 (d, J = 2.4 Hz, 1H, H-4), 3.64

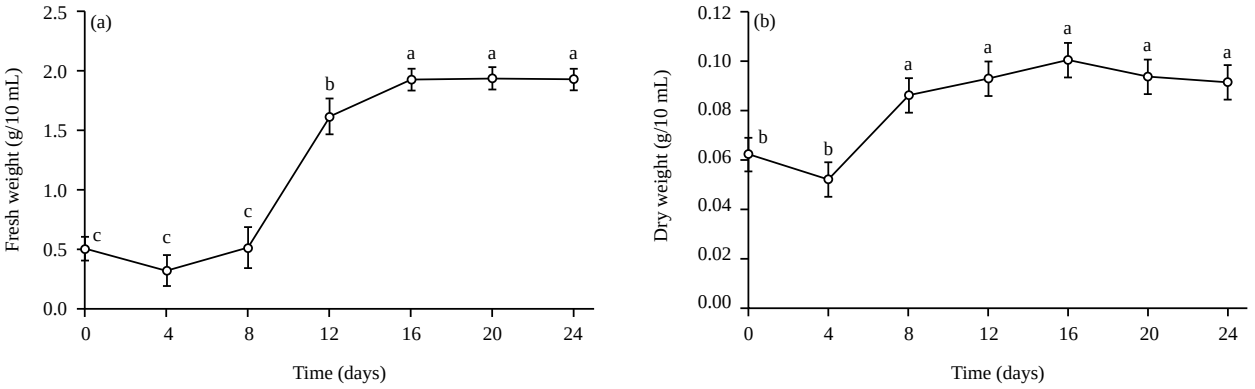

Fig. 1(a-b): Growth kinetics of cells in suspension cultures of *Dalbergia congestiflora* pittier cultured on MST10 (1.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA), (a) Fresh weight and (b) Dry weight

Data represent mean±SD. Different letters indicate significant differences ($p \leq 0.05$, n = 3, Tukey's test)

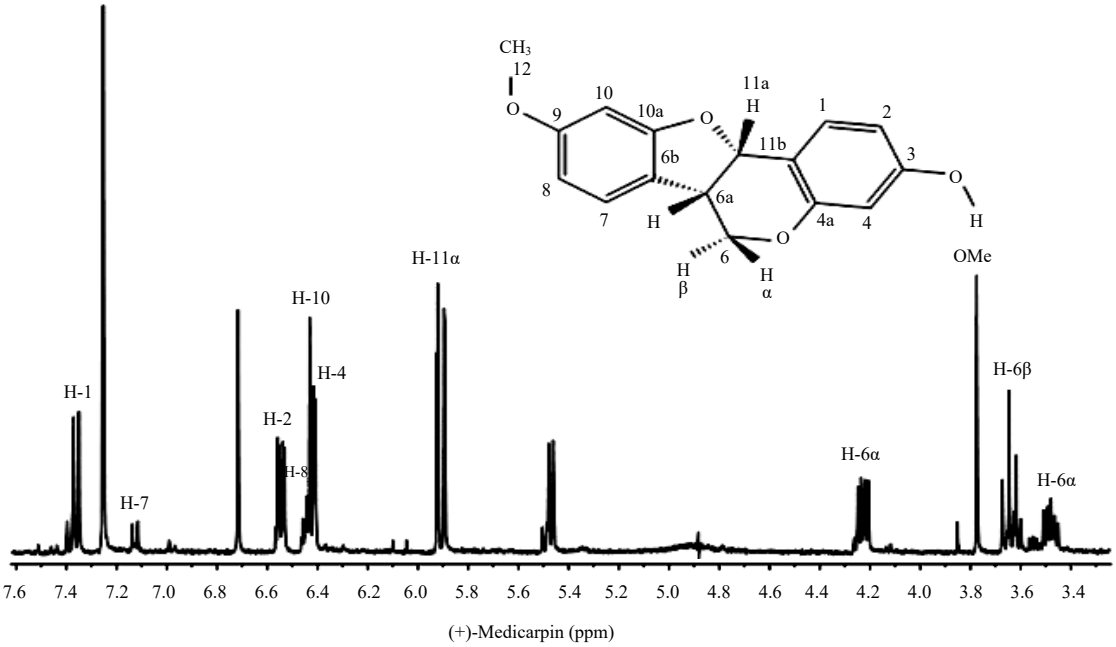

Fig. 2: ^{1}HNMR spectra of (+)-Medicarpin obtained from cell suspensions cultures of *Dalbergia congestiflora* pittier

Table 1: Effect of BAP and NAA combinations to callus induction in leaf explants of *Dalbergia congestiflora* pittier fresh and dry weights were obtained at 30 days of culture

Treatments	BAP (mg L^{-1})	NAA (mg L^{-1})	Fresh weight (g)	Dry weight (g)
1	0.1	0.0	1.1±0.18^{e}	0.3±0.027^{c}
2	0.1	0.1	1.4±0.16^{d}	0.2±0.022^{d}
3	0.25	0.0	2.1±0.11^{b}	0.4±0.05^{b}
4	0.25	0.1	1.5±0.18^{d}	0.3±0.018^{c}
5	0.5	0.0	1.1±0.15^{e}	0.4±0.049^{b}
6	0.5	0.1	1.5±0.14^{d}	0.5±0.038^{b}
7	1.0	0.0	1.8±0.2^{c}	0.5±0.048^{b}
8	1.0	0.1	1.4±0.18^{d}	0.3±0.022^{c}
9	1.5	0.0	1.7±0.16^{c}	0.5±0.045^{b}
10	1.5	0.1	2.3±0.16^{a}	0.8±0.055^{a}
11	2.0	0.0	1.2±0.14^{e}	0.3±0.021^{c}
12	2.0	0.1	0.9±0.11^{e}	0.2±0.014^{d}

Data represent mean±SD, n: Different letters indicate significant differences in each column of weight ($p \leq 0.05$, n = 6, Tukey's test)

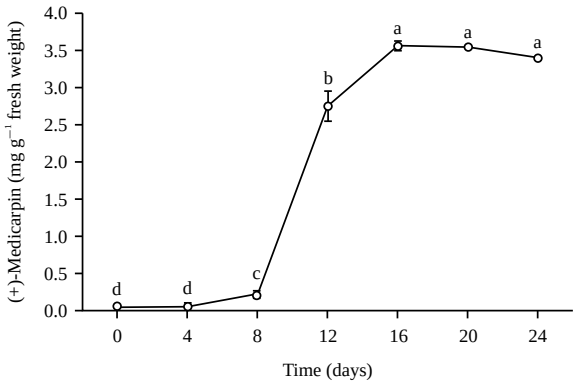

Fig. 3: (+)-Medicarpin accumulation in cell suspensions of *Dalbergia congestiflora* pittier cultured in liquid medium MST10

Each value represented the mean of 3 replicate±deviations

(t, J = 11.0 Hz, 1H, H-6β), 3.52 (ddd, J = 13.9, 7.0, 3.0 Hz, 1H, H-6a), 7.13 (d, J = 8.7 Hz, 1H, H-7), 6.43 (s, 1H, H-10), 5.47 (d, J = 6.8 Hz, 1H, H-11a), 3.77 (s, 3H, OMe).

Accumulation of (+)-medicarpin in cell cultures: The accumulation of (+)-Medicarpin in cell suspensions of *D. congestiflora* was observed mainly during the active phase of growth (day 12) and with a maximum content in the early stationary phase (day 14), with 2.75 and 3.56 mg (+)-Medicarpin/g FW, respectively (Fig. 3).

DISCUSSION

Callus cultures on MST10 medium produced the highest values of biomass compared with other treatments and was selected to grow callus and to establish cell suspensions of *D. congestiflora*. A higher concentration of cytokinin promoted a higher growth of callus, which is consistent with reports for *D. sissoo*, species for which a higher concentration of cytokinin relative to auxins was used[14]. However, in other species of *Dalbergia*[22] and of other genera[13], the induction of callus has been achieved using a higher amount of auxin in comparison to cytokinin. Those factors involved in the endogenous concentrations of phytohormones are yet to be elucidated, because of that explants have been taken from hypocotyl, cotyledons, or embryos, meanwhile in this workleaf explants were used from juvenile shoots.

Dalbergia congestiflora cell suspensions cultured in MST10 medium reached a maximum biomass after 16 days of culture with a four-fold increase compared to the initial inoculum. The time of maximum cell growth depends on the plant species, cell suspensions of *Eucalyptus cinerea* needed 30 days of culture to have a three-fold growth compared to the initial inoculum[13]. These results indicate that cell suspensions should be subcultured at intervals no longer than 16 days for optimal growth.

Free cells, cell division and 80% of cell viability were present in the cell suspensions of *D. congestiflora*, since the first culture and during growth kinetics. The above-mentioned characteristics are a requirement for the establishment of cell suspension cultures as described by Pierik[23], who reports that the presence of free cells, with a viability percentage above 70%, indicates an optimal cell suspensions culture.

After comparing the ^{1}HNMR reported data from Deesamer *et al.*[24] and Martínez-Sotres[4], the mass spectra both callus and cell suspensions of *D. congestiflora* showed several signals similar and consistent in displacement shifts and coupling constants with the isolated medicarpin and the results reported from the heartwood of *D. congestiflora*. Therefore, the results demonstrate the occurrence of (+)-Medicarpin in the obtained callus and cell suspensions for each developed assay.

The content of (+)-Medicarpin in callus of *D. congestiflora* was 1.7 mg g^{-1} FW at 24 days of culture, but the accumulation in cell suspensions was twice than callus culture, so these cellular systems are an excellent prospect for *in vitro* production of this pterocarpan phytoalexin.

This is the first report of *in vitro* production of (+)-Medicarpin in *Dalbergia* spp. cells, the accumulation of this compound has been reported in callus of jack bean (*Canavalia ensiformis*) and white clover (*Trifolium repens*) and in cell suspensions of alfalfa (*Medicago sativa*) and barrel medick (*M. truncatula*), with a content of less than 0.5 µg g^{-1} FW induced by elicitors[25,26]. However, some cell cultures produce low levels of phytoalexin without elicitors as silymarin and forskolin accumulation in cell suspensions of *Silybum marianum* and *Coleus forskohlii*[27].

CONCLUSION

A rapid and effective induction callus method and cell suspension cultures were established for *D. congestiflora* by using leaf explants from shoot tip segments. Callus and cell suspension cultures of *D. congestiflora* provided a material for the (+)-Medicarpin production. Cell growth and (+)-Medicarpin content were higher at 16 days of cell suspension culture, considered an optimal production.

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SIGNIFICANCE STATEMENT

This study reports for the first time the (+)-Medicarpin production in callus and cell suspensions of *Dalbergia congestiflora*. This study suggests that cell suspension cultures of *D. congestiflora* represent an effective system for the production of (+)-Medicarpin, a bioactive compound of considerable interest by biological activity. The results of this study show the (+)-Medicarpin production without the elicitors' addition to culture medium, that before was not found by other researchers. Thus, new studies are necessary to determine the effect of elicitors addition in *D. congestiflora* cell suspensions, a higher production of (+)-Medicarpin could be obtained.

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