

CALLUS INDUCTION IN LEAF EXPLANTS OF *Cissus verticillata* (L.) NICOLSON & C. E. JARVIS

INDUÇÃO DE CALOS EM EXPLANTES FOLIARES DE *Cissus verticillata* (L.) NICOLSON & C. E. JARVIS

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ABSTRACT

Cissus verticillata (L.) Nicolson & C. E. Jarvis is a perennial plant native to the Amazonian Rainforest. This species is used in Brazilian folk medicine for the treatment of diabetes, which has motivated several botanical, chemical and pharmacological studies. The objective of this research was to develop a protocol for callus induction in leaf explants of *C. verticillata*, aiming to provide support for further establishment of cell suspension systems for *in vitro* production of secondary metabolites. The leaves were cut into 1 cm² explants, individually inoculated in test tubes on Murashige & Skoog culture medium supplemented with 3.0% sucrose, 0.6% agar, 2,4-D (0, 1, 2, and 4 mg L⁻¹) and BA (0, 1, 2, and 4 mg L⁻¹) in factorial combinations. On the 42nd day after inoculation, callus induction, percentage of the leaf area covered by callus cells (%LACC), and fresh weight of the explants were evaluated. No callus induction was observed without growth regulators. All the other treatments resulted in 100% induction. However, 2,4-D caused tissue necrosis in all the explants. In the media supplemented with BA and in the absence of 2,4-D, explants did not necrose and callus growth occurred. Highest percentages of LACC were observed in the media supplemented with 1.0 and 4.0 mg L⁻¹ BA, in which all the explants presented 100% of the explant area covered by callus cells. The greatest fresh mass, 18.19 g, was achieved with 4.0 mg L⁻¹ BA in the culture medium.

Index terms: Callogenesis, medicinal plant, secondary metabolites.

RESUMO

Cissus verticillata (L.) Nicolson & C. E. Jarvis é uma planta perene nativa da Floresta Amazônica. Possui hábito herbáceo, escandente ou trepador. É utilizada na medicina popular brasileira para o tratamento de diabetes, o que tem motivado diversos estudos botânicos, químicos e farmacológicos. O objetivo desta pesquisa foi desenvolver um protocolo para indução de calos em explantes foliares de *C. verticillata*, visando subsidiar o futuro estabelecimento de sistemas de células em

suspensão para a produção de metabólitos secundários *in vitro*. As folhas foram segmentadas em explantes de 1 cm², os quais foram individualmente inoculados em tubos de ensaio contendo meio de cultura Murashige & Skoog suplementado com 3,0% de sacarose, 0,6% de ágar, 2,4-D (0, 1, 2 e 4 mg L⁻¹) e BAP (0, 1, 2 e 4 mg L⁻¹) em combinações fatoriais. No 42^o dia após a inoculação, foi avaliada a indução de calos, a porcentagem da área foliar coberta por células de calo (%AFCC) e o peso fresco dos explantes. Não se observou indução de calos sem reguladores de crescimento. Os demais tratamentos resultaram em 100% de indução. Porém, 2,4-D causou necrose tecidual em todos os explantes. Nos meios suplementados com BAP e na ausência de 2,4-D, os explantes não necrosaram e os calos cresceram. As maiores porcentagens de AFCC foram observadas nos meios suplementados com 1,0 e 4,0 mg L⁻¹ de BAP, nos quais todos os explantes apresentaram 100% da área do explante coberta por células de calo. A maior massa, 18,19 g, foi obtida com 4,0 mg L⁻¹ de BAP no meio de cultura.

Termos para indexação: Calogênese, planta medicinal, metabólitos secundários.

INTRODUCTION

Cissus verticillata (L.) Nicolson & C. E. Jarvis is a perennial plant native to the Amazonian Rainforest. Its habit is herbaceous, scandent or climbing (LORENZI & MATOS, 2008). In Brazil, it is colloquially known as “insulin” (BRAGA et al., 2011) due to its use in the Brazilian folk medicine for the treatment of diabetes, which has motivated several botanical, chemical and pharmacological studies (BELTRAME et al., 2001; PEPATO et al., 2003; BARBOSA-FILHO et al., 2005). Its hypoglycemic action was confirmed under laboratory

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conditions by the administration of the tea made from its leaves to normoglycemic rats (BARBOSA et al., 2002). It is also employed in cases of abscesses, muscle inflammation, epilepsy, stroke, hypertension, and used to promote blood circulation (VASCONCELOS et al., 2007). In relation to its antifungal activity, studies have shown the potential of the hydroalcoholic extract of *C. verticillata* leaves against four *Candida* species (BRAGA et al., 2011).

The use of phytotherapy as an alternative to synthetic medicines and the increasing investment in research towards new active principles demand studies on the establishment of *in vitro* cell suspension cultures for the production of relevant substances from a pharmacological standpoint (ARNALDOS et al., 2001; LIMA, 2008; MORAIS et al., 2012). Secondary metabolites can be produced in significant amounts in cell suspension systems, often surpassing the levels achieved by intact plants, without agricultural problems such as seasonality and the need for pesticides, among others. Moreover, production in these systems can be maximized by genetic breeding and operational adjustments of the *in vitro* conditions (MORAIS et al., 2012; OLIVEIRA et al., 2009; CID, 1998).

The objective of this research was to develop a protocol for callus induction in leaf explants of *C. verticillata*, aiming to provide support for the establishment of cell suspension systems for *in vitro* production of secondary metabolites.

MATERIAL AND METHODS

The experiments were carried out at the Laboratory of Plant Tissue Culture of Embrapa, in Porto Velho, Rondonia, Brazil. Leaves were collected from 12 month old plants cultivated under greenhouse conditions, 30% shading and watering three times a day. At the laboratory, they were washed with running tap water and a detergent agent for five minutes and then immersed in 70% (v/v) ethanol for one minute and soaked in a 5.0% (w/v) calcium hypochlorite solution for 30 minutes, and then rinsed three times with sterile water. Explants were produced

by cutting the leaves into 1 cm² pieces in sterile Petri dishes. The leaf explants were individually inoculated with the adaxial surface up in test tubes containing 10.0 mL of MS (MURASHIGE & SKOOG, 1962) medium supplemented with 3.0% (w/v) sucrose, 0.6% (w/v) agar, and factorial combinations of the growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) (0.0, 1.0, 2.0, and 4.0 mg L⁻¹) and benzylaminopurine (BA) (0.0, 1.0, 2.0, and 4.0 mg L⁻¹), totaling 16 treatments. The pH of the medium was adjusted to 5.8 before addition of agar followed by autoclaving at 121°C for 20 minutes. All the cultures were incubated in a growth chamber at 24±1°C under 50 µmol.m⁻².s⁻¹ photosynthetic photon flux density light provided by cool white fluorescent tubes, with a 16 h.d⁻¹ photoperiod. Treatments were arranged in a completely randomized design, each one of the 16 treatments with four replications, each replication represented by four test tubes with one explant, totaling 256 explants. The evaluations of callus induction, percentage of the leaf area covered by callus cells (%LACC), and fresh weight of the explants were performed 42 days after inoculation. Each explant was carefully cleaned with tissue paper and weighed in an analytical scale. Three evaluators attributed scores of 1, 2, 3, and 4 to explants whose leaf area was covered by callus cells in the proportions of 0-25%, 26-50%, 51-75%, and 76-100%, respectively (LAMEIRA et al., 1997). All collected data were submitted to analysis of variance, and the means were compared by Tukey's test ($P \leq 0.05$). Analyses were carried out by using the Biostat 5.0 statistical program.

RESULTS AND DISCUSSION

Seven days after inoculation all concentrations of 2,4-D and BA caused tumescence in 100% of the explants, which was not observed in the treatment without growth regulators. However, 2,4-D caused a toxic effect with tissue necrosis in all the explants, which started at the portion in contact with the media. In all the treatments where this growth regulator was not present no necrosis was observed, including the experimental control.

At 14 days after inoculation no callus induction was observed in the control treatment. All the other treatments resulted in 100% induction. Necrosis in 2,4-D treatments increased considerably, impeding the growth of the callus. In the media supplemented with BA and in the absence of 2,4-D, explants did not necrose and the callus growth was noticeable.

On the 42nd day after inoculation, no necrosis or callogenesis was observed on the experimental control. The explants were still green, without alterations. The explants and callus that had been grown in media supplemented with 2,4-D necrosed completely. The utilization of BA in all concentrations, in the absence of 2,4-D, resulted in induction and growth of friable white callus (Figure 1). Specifically in relation to callus induction, the treatments with growth regulators did not differ, for all of them caused 100% of callus induction (Table 1).

In general, slightly similar concentrations of auxin and cytokinin in the culture medium promote callus induction, but the responses to the interaction of these classes of growth regulators can vary according to the regulator, explant and genotype peculiarities (CORDEIRO et al., 2007). They can act together in synergistic interaction

or not, leading to the dedifferentiation (SANTOS, 2001). However, in this study the highest percentages of LACC were observed in the media supplemented with 1.0 and 4.0 mg L⁻¹ BA, in which all the explants had 100% of the explant area covered by callus cells.

Rodrigues & Almeida (2010) also observed a positive effect of BA alone on callus induction in leaf explants of *Cissus sicyoides* L. in MT (MURASHIGE & TUCKER, 1969) solid medium. The researchers supplemented the medium with 1.0 mg L⁻¹ NAA and 2.0, 4.0, 6.0, and 12.0 mg L⁻¹ BA. The concentration of 4.0 mg L⁻¹ BA promoted the highest number of compact callus while the highest number of friable callus was achieved with 12.0 mg L⁻¹ BA. Nevertheless, the concentration of 6.0 mg L⁻¹ BA resulted in the highest number of explants with 100% of their area covered by callus cells.

Lima et al. (2008) observed the opposite in a study on callus induction in leaf explants of *Croton urucurana* Baill. The authors studied the interaction of different concentrations and combinations of 2,4-D, TDZ, BA and NAA and observed that the use of BA without combination or with NAA did not promote callogenesis, while 2,4-D alone resulted in callus induction with the highest callus fresh mass.



FIGURE 1 – Callus induced in leaf explant of *C. verticillata* on MS medium with 4.0 mg L⁻¹ BA, 42 days after inoculation.

TABLE 1 – Percentages of callus induction and leaf area covered by callus cells (%LACC) in leaf explants of *C. verticillata* submitted to factorial combinations of 2,4-D and BA in MS medium, 42 days after inoculation.

BA (mg L ⁻¹)	2,4-D (mg L ⁻¹)	Callus induction (%)*	%LACC			
			0-25%	26-50%	51-75%	76-100%
0	0	0 b	0 e	0 h	0 g	0 d
0	1	100.0 a	0 e	58.8 bc	41.2 c	0 d
0	2	100.0 a	0 e	94.4 a	5.5 gh	0 d
0	4	100.0 a	0 e	100.0 a	0 f	0 d
1	0	100.0 a	0 e	0 h	0 f	100.0 a
1	1	100.0 a	0 e	11.8 f	70.6 a	17.6 c
1	2	100.0 a	5.9 de	58.8 bc	29.4 d	5.9 d
1	4	100.0 a	37.5 b	43.8 d	12.5 fg	6.3 d
2	0	100.0 a	0 e	0 h	18.8 ef	81.2 b
2	1	100.0 a	1.7 c	66.7 b	11.1 fg	5.6 d
2	2	100.0 a	33.3 b	40.0 d	26.7 de	0 d
2	4	100.0 a	50.0 a	50.0 cd	0 f	0 d
4	0	100.0 a	0 e	0 h	0 f	100.0 a
4	1	100.0 a	0 e	0 h	23.5 de	76.5 b
4	2	100.0 a	6.2 de	6.2 fg	62.6 ab	25.0 c
4	4	100.0 a	14.3 cd	28.6 e	57.1 b	0 d

*Averages followed by the same letter within the same column do not differ by Tukey's test at 5.0%.

Santos et al. (2010) utilized factorial combinations of NAA and IBA to induce callus in leaf explants of *Coffea canephora* cv. Conilon. The authors observed a positive interaction between the two regulators and reported that the half strength MS medium supplemented with 2.0 mg L⁻¹ NAA + 5.0 mg L⁻¹ IBA resulted in callogenesis in all the explants and the highest LACC.

Thomé et al. (2004) studied the micropropagation of *Kalanchoe blossfeldiana* Poelln. and observed 100% of callogenesis in leaf explants by using 1.0 mg L⁻¹ BA + 0.03 mg L⁻¹ NAA with subsequent organogenesis in two cultivars, Gold Trike and Klabat.

Considering the convergence of certain data obtained in relation to the percentage of callus induction and the LACC, the fresh mass was studied in order to access information about the callus cell proliferation. The greatest fresh mass, 18.19 g, was achieved with

4.0 mg L⁻¹ BA in the culture medium (Table 2). The supplementation with 1.0 and 2.0 mg L⁻¹ BA resulted in 14.03 and 12.33 g of fresh mass, respectively.

The highest fresh weight of callus induced in leaf explants and lateral buds of *Ducrosia anethifolia*, a medicinal plant native to Iran, was achieved by Kermanshahi et al. (2012) in MS medium supplemented with 2.0 mg L⁻¹ NAA + 1.0 mg L⁻¹ BA. The fresh mass of callus obtained from leaf explants of *Cleome viscosa* was evaluated by Anburaj et al. (2011). The authors achieved the highest fresh mass in MS medium supplemented with 2.0 mg L⁻¹ IAA. Porto et al. (2014) evaluated the fresh mass of callus from cotyledon leaves of *Sthypnodendron adstringens* (Mart.) Coville and observed the highest weights in MS medium with 0.5 mg L⁻¹ TDZ and 2.0 mg L⁻¹ KIN + 0.5 mg L⁻¹ Picloram.

Table 2 – Total fresh mass of *C. verticillata* explants submitted to factorial combinations of 2,4-D (0.0, 1.0, 2.0 and 4.0 mg L⁻¹) and BA (0.0, 1.0, 2.0 and 4.0 mg L⁻¹), 42 days after inoculation.

BA (mg L ⁻¹)	2,4-D (mg L ⁻¹)	TOTAL FRESH MASS (g)*
0	0	0.91 g
0	1	5.42 cdef
0	2	3.95 ef
0	4	2.78 fg
1	0	14.03 b
1	1	7.81 c
1	2	6.09 cde
1	4	5.51 cdef
2	0	12.33 b
2	1	5.22 cdef
2	2	4.63 def
2	4	3.39 efg
4	0	18.19 a
4	1	11.82 b
4	2	7.62 cd
4	4	5.73 cdef

*Averages followed by the same letter within the same column do not differ by Tukey's test at 5.0%.

CONCLUSIONS

BA and 2,4-D are effective for callus induction in leaf explants of *Cissus verticillata*, however, 2,4-D causes necrosis of the explants, which impedes the callus growth. The utilization of 4.0 mg L⁻¹ BA results in high callus proliferation, covering 100% of the explant area, with an average of 18.19 grams of fresh mass in 42 days of cultivation.

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