Original Article

INDUCTION AND GROWTH CURVE OF CALLI FROM LEAF AND NODAL EXPLANTS OF GENIPAP

INDUÇÃO E CURVA DE CRESCIMENTO DE CALOS OBTIDOS DE EXPLANTES FOLIARES E NODAIS DE JENIPAPEIRO

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ASBTRACT: The aim of this study was to determine the effect of the auxin 2,4-D (2,4-dichlorophenoxyacetic) in calli formation from leaf and nodal segments of genipap and to characterize its growth curve. Explants obtained from shoots previously established from in vitro seedlings were used for calli induction. The experimental design was completely randomized in a 3x5x2 factorial with three accessions (NB, SA, SAL), five concentrations of 2,4-D (0.0; 2.0; 4.0, 6.0 or 8.0 mg L^{-1}) and two times of measurement for calli fresh weight (30 and 60 days). There was callus formation in all treatments tested. It was observed that the best response for callus induction from leaf segments was with 2.0 mg L^{-1} of 2,4-D. For the nodal segment, the response among the accessions was different due to 2,4-D concentrations. The growth curve was plotted according to the fresh weight of callus obtained at intervals of 10 days up to 60 days. Through the established growth curve, the nodal-derived calli from accession SA should be transferred to a new medium, after 40 days of culture.

KEYWORDS: Callogenesis. Plant growth regulators. *Genipa americana* L.

INTRODUCTION

The species *Genipa americana* L. (Rubiaceae) commonly known for geninap, is present in practically all Brazilian regions, from Northeast to the states of São Paulo and Paraná, adapting to the most varied edaphoclimatic conditions (ZAPPI, 2015). It is a species with socioeconomic importance for its woody, food and phytochemical potential (BESSA et al., 2013; SANTOS et al., 2011).

The ability of plants to produce and accumulate secondary metabolites has been decisive to develop technologies based on the in vitro culture of cells, tissues and organs (OKSMAN-CALDENTEY; INZÉ, 2004). Undifferentiated cells, or calli, have been used in studies focused on the production of bioactive compounds, but could also be a useful model to study the first steps towards their regulation and biosynthesis (VASCONCELOS et al., 2012).

Many factors interfere with calli induction under controlled conditions, including those related to the donor plant (e.g. genotype), explant type and medium composition. During the in vitro culture, different explants may be used, but youngest tissues have a greater ability to express totipotency, considering their morphogenetic plasticity (GRATTAPAGLIA; MACHADO, 1998).

Usually, an exogenous supply with plant growth regulators, mostly auxins and cytokinins, is necessary to stimulate cell proliferation (NOGUEIRA et al., 2007). This hormonal balance determines whether cell differentiation will occur and which regenerative route it will follow. Therefore, the physiological effect on the explant depends, mainly on its concentration in the culture medium (TERMIGNONI, 2005).

The knowledge about calli growth dynamic, together with in vitro culture, may support the establishment of optimized protocols, suggesting the most suitable moment for transferring the material to a fresh medium (VASCONCELOS et al., 2012). Four different stages were verified through the growth curve: lag, exponential, stationary and decline (GEORGE, 2008).

The aim of the present work was to determine the best concentration of 2,4-D (2,4-dichlorophenoxyacetic) in callus induction from leaf and nodal segments of three accessions of *G. americana* helded in vitro and to characterize their growth curve.

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MATERIAL AND METHODS

The experiment was performed at the Plant and Tissue Culture Laboratory (LCTP) of Embrapa Tabuleiros Costeiros, Aracaju, Sergipe, Brazil.

Plant material

Accessions NB (Núcleo Bandeirante, Brasília, Distrito Federal), SA (Sabinópolis, Siriri, Sergipe) and SAL (Salvaterra, Ilha de Marajó, Pará) of the Germplasm Active Bank (BAG Jenipapo) kept in vitro were used in this experiment. Nodal segments obtained from pre-establish plantlets were subculture in 30 mL of MS (MURASHIGE; SKOOG, 1962) with 3% of sucrose, 1.0 mg L^{-1} of BAP (6-benzylaminopurine) and 0,7% g L^{-1} of agar. The pH was adjusted to 5.7 ± 0.1 with KOH or HCl prior to autoclaving for 20 min (121ºC). The cultures were maintained in a growth room under a 16 h photoperiod with 52 $mmol.m^{-2}.s^{-1}$ irradiance provided by white fluorescent lamps at 25 ± 2ºC. After 90 days, shoots were used as source of leaf and nodal explants for calli induction.

Calli induction and growth curve

Calli were induced from 0.25 cm² leaf discs and 1.0 cm nodal segments of NB, SA and SAL obtained from in vitro shoots. Explants were inoculated in Petri dishes (50x10 mm) with 20 mL of MS medium, 3% of sucrose and 0.4% of Phytagel®, and supplemented with 0.0; 2.0; 4.0, 6.0 or 8.0 mg L^{-1} of 2,4-D. For the leaf explant, the same concentrations of 2,4-D were used, along with 1.77 mg L^{-1} of BAP, according previous results (ALMEIDA et al., 2015). At 30 and 60 days, calli fresh biomass was weight.

The growth curve was established by determining the fresh weight of calli from the day of inoculation (time 0) at 10-day intervals up to 60 days, which correspond to first cultivation period, considering the auxin available in the culture medium (SACHS, 1991). The percentage of calli growth was determined from the equation (VASCONCELOS et al., 2012):

Calli growth (%) = [(FWf – FWi)/(FWf)] * 100

Whereas FWi is the initial fresh weight and FWf is the final fresh weight of calli in grams (g). The curve was plotted from the average of three replicates with two explants of each time (0, 10, 20, 30, 40 50 and 60 days).

Statistical analysis

A completely randomized design in a triple factorial (3x5x2) was used, with five replications, each one composed by a Petri dish with two explants. The treatments consisted of three accessions (NB, SA, SAL), five concentrations of 2,4-D (0.0; 2.0; 4.0, 6.0 or 8.0 mg L^{-1}), and two times of measurement for calli fresh weight (30 and 60 days). Data (fresh weight) was subjected to the analysis of variance (ANOVA) and means compared by Tukey test ($p < 0.05$). For 2,4-D concentrations, regression equations were estimated. The statistical analysis was conducted using SISVAR (FERREIRA, 2014). The graphs were created with Microsoft Excel.

RESULTS AND DISCUSSION

Calli induction and growth curve from leaf explants

There was a significant effect of the triple interaction for the fresh weight of calli obtained from leaf explants of genipap. For all the treatments, the concentration of 2.0 mg L^{-1} of 2,4-D promoted higher values on fresh weight of calli, at 30 and 60 days of culture. Accession SA showed a production of 0.2201 g of calli biomass, which differed statistically from NB and SAL, at 60 days (Table 1).

At 60 days of culture, the behavior of the variable calli weight was cubic for NB, SA and SAL. For NB, the fresh weight induction in the presence of 2.22 mg L^{-1} of 2,4-D was 0.1128 g and for SAL, 0.1491 g in the concentration of 1.98 mg L^{-1} of 2,4-D. The accumulation of fresh weight in the concentration of 2.77 mg L^{-1} was 0.1759 g to SA. A reduction on fresh weight of calli in concentrations higher than 4.0 mg L^{-1} of 2,4-D was verified for all accessions and must be related to explant oxidation or necrosis. Growth regulators may be toxic for plant tissues in excessive concentrations, as noted in the callus formation of *Tridax procumbens* L. (CERQUEIRA et al., 2002) and *Cissus verticillata* (L.) Nicolson & C. E. Jarvis (SANTOS et al., 2014).

The balance between auxin and cytokinin tends to be efficient in calli induction. The best response of callus induction in leaf explants of *Copaifera langsdorffii* Desf. was obtained through MS supplemented with 2.0 mg L^{-1} of 2,4-D and 1.0 mg L-1 of BAP, in which a biomass accumulation of 0.0092 g was verified (AZEVEDO, 2003). However, the higher percentage of callus formation in leaf explants of *G. americana* L. was noticed in the presence of 8.0 mg L^{-1} of 2,4-D (89.50%) or 2.0 mg L^{-1} of BAP (85.29%) for SIR accession (ALMEIDA et al., 2015).

Table 1. Fresh weight of calli from leaf explants of NB, SA and SAL after 30 and 60 days in MS medium supplemented with different concentrations of 2,4-D.

Time (days)	2,4-D (mg L^{-1})	NB	SA	SAL
30	0.0	0.0137 Aa	0.0207 Aa	0.0170 Aa
	2.0	0.0321 Bb	0.0692 Ba	0.0341 Bb
	4.0	0.0277 Aa	0.0153 Aa	0.0205 Ba
	6.0	0.0311 Aa	0.0157 Aa	0.0262 Ba
	8.0	0.0210 Aa	0.0162 Aa	0.0237 Aa
	Equations	Ns	$0.0011x^3 - 0.0135x^2 + 0.0395x + 0,0238$	ns
	R^2	-	0.7108	-
		NB	SA	SAL
60	0.0	0.0183 Aa	0.0271 Aa	0.0201 Aa
	2.0	0.1353 Ac	0.2201 Aa	0.1861 Ab
	4.0	0.0450 Aab	0.0289 Ab	0.0674 Aa
	6.0	0.0467 Ab	0.0278 Ab	0.1215 Aa
	8.0	0.0317 Aa	0.0167 Aa	0.0256 Aa
	Equations	$0.002x^3 - 0.0269x^2 + 0.0898x + 0.0241$	$0.0039x^3 - 0.0506x^2 + 0.1545x + 0.0381$	$0.0014x^3 - 0.0231x^2 + 0.0958x + 0.0312$
	R^2	0.7204	0.7191	0.5566

*Means followed by the same lowercase letter in the line compares accessions within concentration of 2,4-D and time and followed by the same uppercase letter in column compares time within accessions and concentration of 2,4-D. Same letters do not differ by Tukey's test ($p < 0.05$).

Calli obtained from leaf segments with the use of this auxin presented a friable texture and non-embryogenic features.

Based on the fresh weight, the presence of two distinct growth phases was observed for leaf-derived callus (Figure 1).

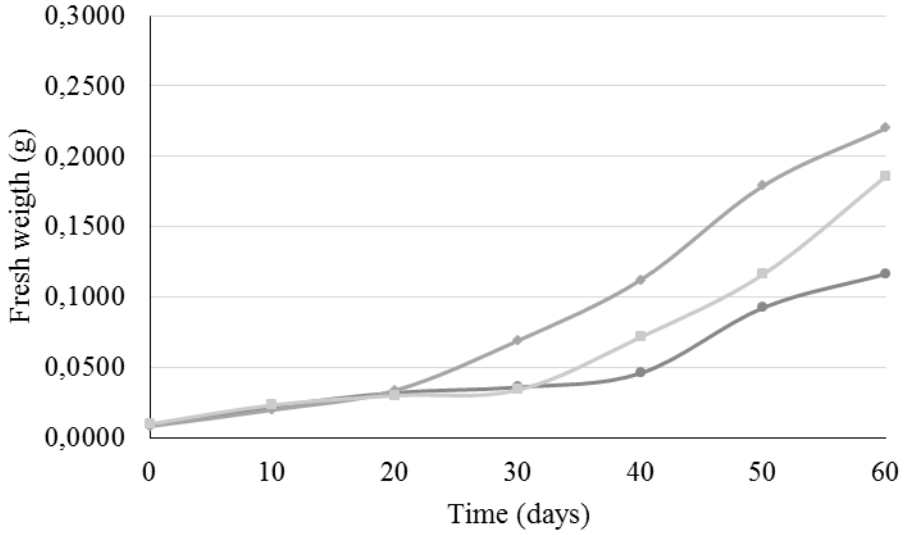


Figure 1. Growth curve of *G. americana* leaf-derived callus of NB (●), SA (♦) and SAL (■) on MS medium supplemented with 2.0 mg L^{-1} of 2,4-D, incubated in the dark. Growth was determined every 10 days after inoculation.

The lag phase occurred up to the 30th day of culture for all accessions. After that, cell growth moved into an exponential phase with a biomass increase of 69.09% for NB, 68.56% for SA and 81.68% for SAL, up to 60 days of culture. The lag phase of calli obtained from leaf segments of *Jatropha curcas* L. occurs up to the day 15 of culture (FEITOSA et al., 2013). However, the occurrence of this phase from leaf segments of *Coffea arabica* cv. Rubi occurs up to the day 42 after the inoculation (SANTOS et al., 2003).

Calli induction and growth curve from nodal explants

There was also significant effect of the triple interaction for the fresh weight of calli obtained from nodal explants (Table 2). The concentration of 4.0 mg L^{-1} of 2,4-D produced 0.4305 g of calli biomass for NB, which differed statistically from SA and SAL, at 60 days.

Table 2. Fresh weight of calli from nodal explants of NB, SA and SAL after 30 and 60 days in MS medium supplemented with different concentrations of 2,4-D.

Time (days)	2,4-D (mg L^{-1})	NB	SA	SAL
	0.0	0.0439 Aa	0.0299 Aa	0.0337 Aa
	2.0	0.1569 Ba	0.0784 Ab	0.1322 Bab
30	4.0	0.1762 Ba	0.1695 Aa	0.1551 Aa
	6.0	0.1086 Aa	0.0892 Aa	0.0383 Aa
	8.0	0.0578 Aa	0.0677 Aa	0.0361 Aa
	Equations	$-0.0074x^2 + 0.0582x + 0.0536$	$-0.0056x^2 + 0.0488x + 0.0252$	$-0.0061x^2 + 0.0443x + 0.0482$
	R^2	0.9005	0.7300	0.6426
		NB	SA	SAL
	0.0	0.0551 Aa	0.0359 Aa	0.0337 Aa
	2.0	0.3679 Aa	0.0987 Ac	0.2330 Ab
60	4.0	0.4305 Aa	0.1539 Ab	0.1948 Ab
	6.0	0.0961 Aa	0.1222 Aa	0.0520 Aa
	8.0	0.0704 Aa	0.0554 Aa	0.0295 Aa
	Equations	$0.0058x^3 - 0.0891x^2 + 0.3417x + 0.0429$	$-0.0062x^2 + 0.0526x + 0.0313$	$0.0037x^3 - 0.0537x^2 + 0.1931x + 0.0376$
	R^2	0.9200	0.9654	0.9964

*Means followed by the same lowercase letter in the line compares accessions within concentration of 2,4-D and time and followed by the same uppercase letter in column compares time within accession and concentration of 2,4-D. Same letters do not differ by Tukey's test ($p < 0.05$).

The time of 60 days favored a greater accumulation of callus biomass (Figure 2). The fresh weight varied according a cubic regression, with a gradual addition of this variable up to the concentrations of 2.55 mg L^{-1} and 2.39 mg L^{-1} of 2,4-D for NB and SAL, which correspond to a biomass accumulation of 0.4310 g and 0.2429 g. For SA, a quadratic behavior was observed, with maximum point of 4.24 mg L^{-1} of 2,4-D (0.1429 g).

The calli formation in nodal segments of genipap had a direct relation with the concentration of 2,4-D and with the time of in vitro culture in induction medium. This results support Almeida et al. (2015), whom observed a percentage of callus formation of 87.50% derived from nodal explants of genipap when cultivated in MS medium, with 4 mg L^{-1} of 2,4-D. In high concentrations of 2,4-D, they also detected calli oxidation and necrosis.

Genotypic differences play a significant role in callus formation, as exhibited in coffee (REZENDE et al., 2011). These differences correspond to the variation in endogenous levels of growth regulators of explants associated to culture conditions that influence the formation and development of the callus (SMITH, 2013). NB accession was classified as a distinct group when compared to other accessions and presented superiority regarding micro-propagation (SÁ, 2014).

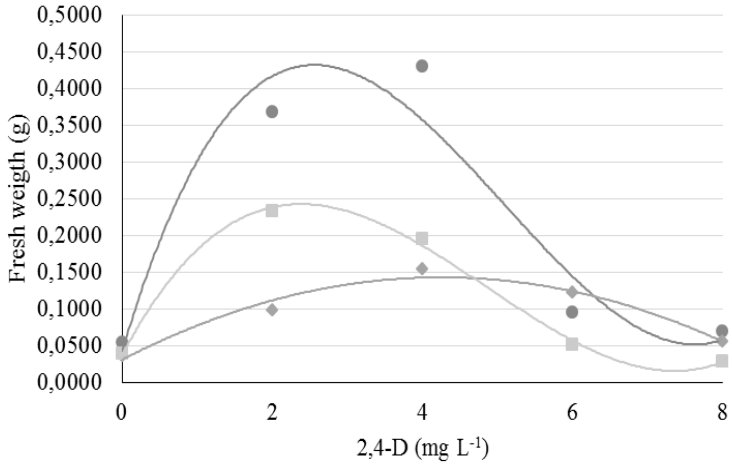


Figure 2. Fresh weight (g) of calli from nodal explants of NB (●), SA (♦) and SAL (■), according to 2,4-D concentrations at 60 days.

Calli obtained from nodal segments with the use of this auxin presented a friable texture and non-embryogenic features. For calli derived from nodal explants, the concentrations of 4.0 mg L^{-1} of 2,4-D was selected for NB and SA and 2.0 mg L^{-1} of 2,4-D for SAL, which correspond to the highest production of callus biomass (Figure 3).

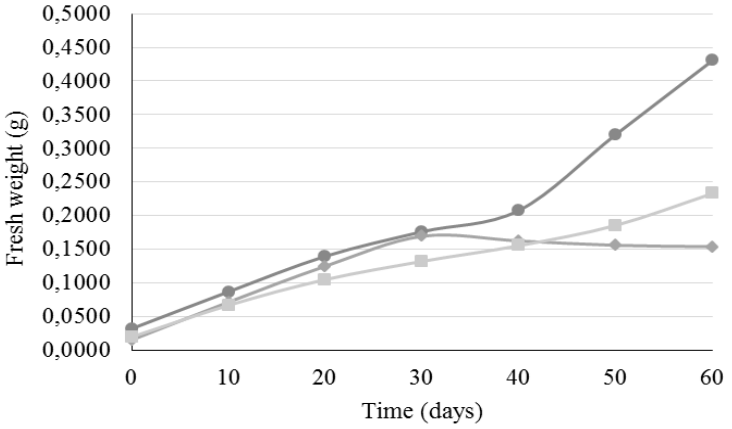


Figure 3. Growth curve of *G. americana* nodal-derived callus of NB (●) and SAL (■) on MS medium supplemented with 4.0 mg L^{-1} of 2,4-D and SA (♦) on MS medium supplemented with 2.0 mg L^{-1} of 2,4-D, incubated in the dark. Growth was determined every 10 days after inoculation.

The growth curve of calli derived from nodal explants presented a distinctive standard between the accessions tested. For NB and SAL, there was a linear behavior. The lag phase, in which explant cells prepare to cell division, occurred up to the 30th day of inoculation. Registered increases were 81.73% for NB and 84.98% for SAL. From this point, a high accumulation of biomass up to day 60 was verified, which represents the exponential growth phase, with a linear increase of 59.07% for NB, and 43.25% for SAL.

Accession SA presented a growth curve with three phases: lag, exponential and stationary. The lag and exponential phases occurred simultaneously up to day 30 after inoculation, with a biomass increase of 90.84%. A stationary period, marked by the reduction on increase of fresh weight up to day 60 of culture, suggests no more biomass synthesis is occurring. Azevedo (2003) considers the calli transfer to new culture mediums must be carried out during this period. For *Myracrodruon urundeuva* Fr. All. calli, this process must occur at day 56 of culture (VASCONCELOS et al., 2012); for *Coffea canephora* L. calli, the transfer must occur at day 70 of culture (SANTOS et al., 2008).

CONCLUSIONS

Calli derived from leaf segments show a slower growth in relation to nodal segments.

The calli induction is possible in leaf segments of genipap in a MS medium with 2.0 mg L^{-1} of 2,4-D. Calli obtained from nodal segments from NB and SA has a better induction response in the concentration of 4.0 mg L^{-1} and for SAL, in 2.0 mg L^{-1} of 2,4-D.

The growth curve of calli derived from leaf explants from NB, SA and SAL presents a linear standard, with two distinct growth phases. Through the growth curve, the nodal-derived calli from SA should be transfer to a new medium, after 40 days of in vitro culture.

RESUMO: O objetivo desse trabalho foi determinar o efeito da auxina 2,4-D (ácido diclorofenoxiacético) na calogênese de segmentos foliar e nodal de jenipapeiro e caracterizar sua curva de crescimento. Explantes obtidos de brotações pré-estabelecidas a partir de plântulas in vitro foram utilizados na indução de calos. O delineamento experimental utilizado foi o inteiramente casualizado em esquema fatorial 3x5x2, com três acessos (NB, SA e SAL), cinco concentrações de 2,4-D (0,0; 2,0; 4,0; 6,0 ou 8,0 mg L^{-1}) e dois tempos de avaliação (30 e 60 dias) da massa fresca de calos. Houve formação de calos em todos os tratamentos testados. Observou-se que a melhor resposta de indução ocorreu na concentração de 2,0 mg L^{-1} para calos oriundos de segmentos foliares. Para o segmento nodal a resposta entre os acessos foi diferenciada em função das concentrações de 2,4-D. A curva de crescimento foi plotada a partir da massa fresca dos calos obtida em intervalos de 10 dias até os 60 dias. Através da curva de crescimento estabelecida, os calos derivados de segmentos nodais do acesso SA devem ser transferidos para um novo meio de cultura, 40 dias após à inoculação.

PALAVRAS-CHAVE: Calogênese. Reguladores de crescimento vegetal. *Genipa americana* L.

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