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Growth pattern of friable calluses from leaves of *Capsicum annuum* var. *annuum* cv. Iberaba Jalapeño[1]

Padrão de crescimento de calos friáveis de folhas de *Capsicum annuum* var. *annuum* cv. Iberaba Jalapeño

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ABSTRACT - The genus *Capsicum* belongs to the Solanaceae botanical family and is notable for the production of secondary metabolites of medicinal and economical importance. *In vitro* methods have been successfully utilized for the large scale production of plant secondary metabolites. The objective of this study was to establish a protocol for callus induction from leaves, nodal and internodal segments of the cultivar *C. annuum* var. *annuum* cv. Iberaba Jalapeño, and to determine the growth pattern of the calluses, aiming at the identification of the deceleration phase, when the callus cells must be subcultured for the establishment of cell suspensions and the production of secondary metabolites. The explants were inoculated into a medium supplemented with BA and 2,4-D in factorial combination. Percentage of callus induction and the explant area covered by callus cells (ACCC) were evaluated. The procedures that resulted in higher proliferation of callus cells were repeated in order to determine the growth curve of the calluses. The highest callus induction percentage and ACCC were observed with the combination of 2.22 μM BA + 18.10 μM 2,4-D for leaf and internodal explants and 2.22 μM BA + 9.05 μM 2,4-D for nodal explants. The calluses produced were friable and whitish, and their growth pattern followed a sigmoid shape. The deceleration phase started on the 31st day of cultivation for leaf explants, on the 26th for internodal explants, and on the 29th day for nodal explants.

Key words: Callogenesis. Growth curve. Secondary metabolites.

RESUMO - O gênero *Capsicum* pertence à família Solanaceae e é notável pela produção de metabólitos secundários de importância medicinal e econômica. Métodos de cultivo *in vitro* têm sido utilizados com sucesso para a obtenção de metabólitos secundários de plantas. O objetivo deste trabalho foi estabelecer um protocolo para a formação de calos *in vitro* a partir de explantes foliares, segmentos internodais e nodais da cultivar *C. annuum* var. *annuum* cv. Iberaba Jalapeño, e determinar sua curva de crescimento, visando à identificação da fase de desaceleração, quando os calos devem ser subcultivados para o estabelecimento de suspensões celulares e produção de metabólitos secundários. Os explantes foram inoculados em meio suplementado com BAP e 2,4-D em combinação fatorial. Foram avaliadas as variáveis de indução de calos (IC) e a área coberta por células de calos (ACCC) do explante. Os tratamentos que resultaram em maior proliferação de células de calos foram repetidos para determinar a curva de crescimento dos calos. O tratamento que resultou em maior porcentagem de IC e ACCC para explante foliar e internodal foi 2.22 μM de BAP + 18.10 μM de 2,4-D; e para o segmento nodal foi 2.22 μM de BAP + 9.05 μM de 2,4-D. Houve formação de calos friáveis e esbranquiçados, e suas curvas de crescimento seguiram um padrão sigmoide. A fase de desaceleração iniciou no 31º dia de cultivo para explante foliar, no 26º dia para explante internodal e no 32º dia para explante nodal.

Palavras-chave: Calogênese. Curva de crescimento. Metabólitos secundários.

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INTRODUCTION

Plant cell and organ cultures have emerged as potential sources of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavors, fragrances, coloring agents, biopesticides, and food additives (MURTHY; LEE; PAEK, 2014).

The genus *Capsicum* belongs to the Solanaceae botanical family (ARROWSMITH *et al.*, 2012) and is cultivated in several countries (RICCI *et al.*, 2013). There are 33 species, of which only five have been domesticated - *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. futescens* L. and *C. pubescens* Ruiz & Pav. (BIANCHETTI, 1996).

The medicinal importance of the *Capsicum* genus has been confirmed by studies involving the detection of capsaicinoids, secondary metabolites of the alcaloid chemical group, and their numerous benefits for human health (SANATOMBI; SHARMA, 2008). The abundance of compounds of agricultural interest such as flavonoids, cumarins, saponins and essential oils have promoted the study of *Capsicum* species as alternatives in the control of parasites (LUZ, 2007).

The concentration of secondary metabolites in a plant varies according to the interactions; plant-animal, plant-plant, and nutritional stresses and, in spite of the existence of a genetic control, the expression can be modified as a result of the interaction of biochemical, physiological and ecological processes (GERSHENZON; ENGELBERTH, 2013).

Secondary metabolites can be efficiently produced *in vitro*. Research to date has succeeded in producing a wide range of valuable secondary phytochemicals in disorganized callus or suspension cultures (HUSSAIN *et al.*, 2012). The major advantages of a cell culture system over the conventional cultivation of whole plants are; (1) useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions; (2) cultured cells would be free of microbes and insects; (3) the cells of any plant could easily be multiplied to yield their specific metabolites; (4) automated control of cell growth and rational regulation of metabolite processes would reduce the labor costs and improve productivity; (5) organic substances are extractable from callus cultures (VANISREE *et al.*, 2004).

To date, no study has described the development of protocols for callus induction in *Capsicum annuum* var. *annuum* cv. Iberaba Jalapeño. The determination of the procedures for callogenesis and the subsequent study of the callus growth pattern are necessary for the establishment of cell suspensions and also to subsidize studies regarding the bioactivity of its secondary metabolites.

The focus of this study is the establishment of a protocol for callogenesis in leaf, nodal and internodal explants of Jalapeño plants, evaluating the effects of different concentrations and combinations of the growth regulators 6-benzylaminopurine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D), and the determination of the callus growth curve, aiming at the identification of the deceleration phase, when the callus cells must be subcultured for the establishment of cell suspensions and the production of active principles of agricultural interest.

MATERIAL AND METHODS

Callus induction

The experiments were carried out at the Plant Tissue Culture Laboratory at Embrapa (Brazilian Agricultural Research Corporation). Seeds of *C. annuum* var. *annuum* cv. Iberaba Jalapeño were purchased at the local market and submitted to disinfestation procedures by washing with running tap water and a detergent agent for five minutes, immersion in 70% ethanol for one minute and in a 1.5% (v/v) sodium hypochlorite solution for 15 minutes, and then rinsed three times with sterile water. In an aseptic hood, the seeds were individually inoculated into test tubes with 10 mL of an MS (MURASHIGE; SKOOG, 1962) basal culture medium supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar, pH 5.8, autoclaved at 121 °C for 20 minutes. After 45 days of cultivation, the plants were approximately 8 cm tall. Under aseptic conditions, the explants were produced; the leaves were cut in explants of 1.0 cm^2, the internodal segments in explants of 1.0 cm length and the nodal segments in explants of 0.03 cm^3, all inoculated individually into test tubes with 10 mL of an MS basal culture medium containing 30 g L^{-1} sucrose and 6 g L^{-1} agar, pH 5.8, autoclaved at 121 °C for 20 minutes. The medium was supplemented with BA (0, 0.44, 2.22 and 11.10 µM) and 2,4-D (0, 4.52, 9.05 and 18.10 µM) in a factorial combination. The explants were incubated in a growth chamber at 26±1 °C under light provided by cool white fluorescent tubes (50 $\mu mol.m^{-2}.s^{-1}$) 16 hours a day. Callus formation was evaluated weekly until the 49th day, by assessing the number of callus induced per treatment and the explant area covered by callus cells (ACCC), according to Mendonça *et al.* (2013). Variance analyses, test F ($P<0.05$) and Scott-Knott test ($P<0.05$) were performed by using the Assistat 7.5 statistical program.

Determination of the growth curve

The explants were individually inoculated into test tubes containing an MS basal culture medium and the hormonal combinations that resulted in the highest callus cell proliferation; 18.10 µM 2,4-D + 2.22 µM BA

for leaf and internodal explants and 9.05 µM 2,4-D + 2.22 µM BA for nodal explants. The explants were incubated in a growth chamber at 26±1 °C under light provided by cool white fluorescent tubes (50 µmol.m^{-2}.s^{-1}), 16 hours a day. In the subsequent 49 days, every seven days, three calluses from each treatment were carefully separated from culture medium and weighed on a precision scale in order to determine their fresh weight. These calluses were kept in an oven at 50 °C until reaching constant weight and then weighed again to determine their dry mass. From these data sets the lag, exponential, linear, deceleration and stationary phases of callus growth were determined.

RESULTS AND DISCUSSION

Callus induction

Callus induction became apparent on the fifth day of culture, with a swelling of the explants. On the 14th day callus formation was observed in all the explants and in all the hormonal combinations, except for the leaf and internodal explants cultivated without BA or 2,4-D; however, nodal explants gave origin to calluses even in the absence of the growth regulators. The calluses produced were friable and whitish. As mentioned by Souza, Berkov and Santos (2014), friable calluses are distinct from compact calluses, as the former are characterized by loosely aggregated cells, with lower density and the latter are thicker aggregates of cells with higher density. The friable calluses have different cell types with different structural and histochemical characteristics, mainly characterized by the presence of cells in rapidly small growing, isodiametric, with high frequency of cell divisions (SOUZA *et al.*, 2011). This kind of callus can be used to initiate cell suspension cultures, for the cells can easily disperse in the liquid medium.

The variance analyses for callus induction percentage and ACCC are summarized in the Tables 1 and 2. The effect of BA and 2,4-D on callus induction and ACCC were highly significant in the three explants, as well as their interaction.

Callus induction occurred in all the explants where BA or 2,4-D were supplemented, alone or in combination, but did not occur in the absence of growth regulators. The interaction between these two growth regulators was positive for callus induction, what was expected, because in general an adequate balance between auxins and cytokinins is needed to the differentiation of specialized cells (SANTOS; SOUZA, 2016).

Table 1 - Results of the analysis of variance for callus induction in explants of Jalapeño plants submitted to different combinations of BA and 2,4-D in the culture medium 28 days after inoculation

Explant	Source of variation	Degrees of Freedom	Mean Square	F
Leaves	BA	3	13.50	14.40**
	2,4-D	3	18.50	19.73**
	BA x 2,4-D	9	4.00	4.26**
	Treatment	15	8.80	9.39**
	Residue	32	0.94	
	Total	47		
Internodal segments	BA	3	6.25	11.11**
	2,4-D	3	10.25	18.22**
	BA x 2,4-D	9	7.08	12.59**
	Treatment	15	7.55	13.42**
	Residue	32	0.56	
	Total	47		
Nodal segments	BA	3	3.83	5.93**
	2,4-D	3	9.00	13.03**
	BA x 2,4-D	9	4.72	7.31**
	Treatment	15	5.40	8.36**
	Residue	32	0.64	
	Total	47		

** - significant at 1% probability by F test

Table 2 - Results of the variance analyses for explant area covered by callus cells in explants of Jalapeño plants submitted to different combinations of BA and 2,4-D in the culture medium, 28 days after inoculation

Explant	Source of variation	Degrees of Freedom	Mean Square	F
Leaves	BA	3	6.67	48.22**
	2,4-D	3	13.24	9.70**
	BA x 2,4-D	9	1.47	10.67
	Treatment	15	4.87	35.18
	Residue	32	0.14	
	Total	47		
Internodal segments	BA	3	2.85	13.20**
	2,4-D	3	8.51	39.43**
	BA x 2,4-D	9	3.89	18.06**
	Treatment	15	4.61	21.36**
	Residue	32	0.21	
	Total	47		
Nodal segments	BA	3	2.02	7.98**
	2,4-D	3	8.05	31.87**
	BA x 2,4-D	9	2.53	10.02**
	Treatment	15	3.53	13.98**
	Residue	32	0.25	
	Total	47		

** - significant at 1% probability by F test

The percentages of callus induction (CI) and percentages of the explant area covered by callus cells (ACCC) in leaf, internodal and nodal explants are summarized in Table 3. The highest percentage of callus induction in leaf explants was observed with combinations of 2,4-D from 4.52 to 18.10 µM and BA from 4.44 to 11.10 µM, or BA in isolation at the concentration of 11.10 µM, which resulted in formation of callus in 100% of the explants. The treatment with 2.22 µM BA + 18.10 µM 2,4-D resulted in the highest ACCC, with 100% of the explant area covered by callus cells.

On the internodal explants, several treatments reached 100% callus induction; 4.52 to 18.10 µM 2,4-D in combination with 0.44 and 2.22 BA, or BA in isolation, at 11.10 µM. Khan *et al.* (2011) studied the effects of 2,4-D and BA on internodal explants of *C. annuum* L. cv. Pusa Jwala and observed 70% callus induction with the use of 2,4-D in isolation at the concentration of 10.0 µM or, in combination to 1.78 µM BA, 95% callus induction. The combination of 18.10 µM 2,4-D + 2.22 µM BA resulted in the highest ACCC on internodal explants.

In relation to nodal explants, callus induction and ACCC were higher in the concentrations ranging from 9.05 to 18.10 µM 2,4-D in combination with 0.44 to 2.22 µM BA, in the combination of 4.52 µM 2,4-D + 0.44 µM BA, or 2,4-D in isolation at the concentrations of 4.52 and 9.05 µM, all of them leading to 100% induction.

The concentration of 11.10 µM BA, without 2,4-D, resulted in shoot formation in addition to callus induction. This is in accordance with Otroshy, Moradi and Khayam Nekouei (2011), who regenerated plantlets of *C. annuum* from nodal explants by using 11.10 µM BA. Ebida and Hu (1993) observed shoot formation in *C. annuum* L. cv. Early California Wonder by using concentrations of BA ranging from 4.44 to 44.44 µM in combination with 0.44 µM 1-naphthaleneacetic acid (NAA). In general, the presence of cytokinins is essential for shoot formation, and even can be satisfactory in isolation, as observed by Singh and Tiwari (2012).

The highest ACCC (100%) on the nodal explants was achieved with the combination of 9.05 µM 2,4-D + 2.22 µM BA. The formation of callus on the nodal explant without the addition of growth regulators can be an effect of a preexisting hormonal balance in the explants (POZO *et al.*, 2005) or also of the physical and chemical injuries to which *in vitro* cultivated tissues commonly pass through (DALPONTE; VALLE; VALLE, 2010).

Evaluating simultaneously the two variables, callus induction and ACCC, the treatments with the highest level of callus cell proliferation were 18.10 µM 2,4-D + 2.22 µM BA for leaf and internodal explants, and 9.05 µM 2,4-D + 2.22 µM BA for nodal explants.

Umamaheswari and Lalitha (2007) tested several kinds of explants; young leaves, buds, pericarp tissue, nodal segments, and placental region, cultivated in an MS medium with gibberellic acid (GA_3), indole-3-acetic acid (IAA), NAA, 2,4-D and KIN in diverse combinations. The placental region submitted to the combination of 9.05 µM 2,4-D + 2.32 µM kinetin (KIN) surpassed all the other treatments in relation to the production of callus and this tissue is being used for the commercial production of capsaicin. The authors also recorded the formation of large and friable calluses in leaf explants of *C. annuum* L. by using a combination of 9.05 µM 2,4-D + 2.32 µM KIN.

Table 3 - Percentages of callus induction (CI) and percentages of the explant area covered by callus cells (ACCC) in leaf, internodal and nodal explants of Jalapeño plants submitted to different combinations of BA and 2,4-D in the culture medium 28 days after inoculation

2,4-D (µM)	BA (µM)			
	0	0.44	2.22	11.10
Percentages of CI in leaf explants				
0.0	0 bC*	40 bB	40 bB	100 aA
4.52	80 aA	100 aA	100 aA	100 aA
9.05	100 aA	100 aA	100 aA	100 aA
18.10	20 bB	100 aA	100 aA	100 aA
Percentages of ACCC in leaf explants				
0.0	0 cA*	10 cA	10 cA	22 bA
4.52	30 bB	77 aA	78 bA	70 aA
9.05	55 aB	50 bB	78 bA	70 aA
18.10	6 cC	75 aB	100 aA	66 aB
Percentages of CI in internodal explants				
0.0	0 bC*	40 bB	40 bB	100 aA
4.52	80 aB	100 aA	100 aA	60 bC
9.05	80 aA	100 aA	100 aA	40 bB
18.10	80 aA	100 aA	100 aA	40 bB
Percentages of ACCC in internodal explants				
0.0	0 cB*	10 bB	15 cB	73 aA
4.52	47 bB	82 aA	77 bA	45 bB
9.05	65 aA	88 aA	80 bA	27 bB
18.10	77 aB	78 aB	100 aA	31.5 bC
Percentages of CI in nodal explants				
0.0	14 bB*	40 bB	80 aA	80 aA
4.52	100 aA	100 aA	80 aA	80 aA
9.05	100 aA	100 aA	100 aA	60 bB
18.10	80 aA	100 aA	100 aA	40 bB
Percentages of ACCC in nodal explants				
0.0	3.5 bC*	16.5 bC	33 cB	58 aA
4.52	73 aA	60 aA	73 bA	63 aA
9.05	73 aB	75 aB	100 aA	50 aC
18.10	75 aA	80 aA	78 bA	15 bB

*Averages followed by the same capital letter do not differ in the same row by Scott-Knott test at 5% probability; averages followed by the same lower case letter do not differ in the same column by Scott-Knott test at 5% probability

Although there was callus induction in the absence of BA or of 2,4-D, the ACCC had the lowest values in these treatments in all the explants, evidencing the necessity of supplementation of the medium with combinations of the two growth regulators for an effective callus cell proliferation, as an outcome of the hormonal balance between cytokinins and auxins.

Callus induction can be dependent on cytokinins and auxins or only one of these classes of growth regulators (SANTOS *et al.*, 2014). Barbosa *et al.* (1994) observed different morphogenic responses to the same concentrations of BA, Thidiazuron (TDZ) and adenine in apical, cotyledonary and hypocotyledonary explants of *C. annuum*. In general, TDZ and BA promoted callus formation; BA in isolation resulted in axillary bud formation.

The growth regulator 2,4-D is the most often used auxin in callogenesis and has been referred to as essential in some cases (SANTOS *et al.*, 2014). The auxins are able to start cell division and to control the processes of growth and cell elongation (NOGUEIRA *et al.*, 2008). In general, slightly similar concentrations of auxins and cytokinins in the culture medium promote callus induction, but the responses to interactions 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 dedifferentiation. These interactions have been used and tested in different forms to establish and to refine the exact concentrations in each situation (SANTOS *et al.*, 2014).

Callus growth

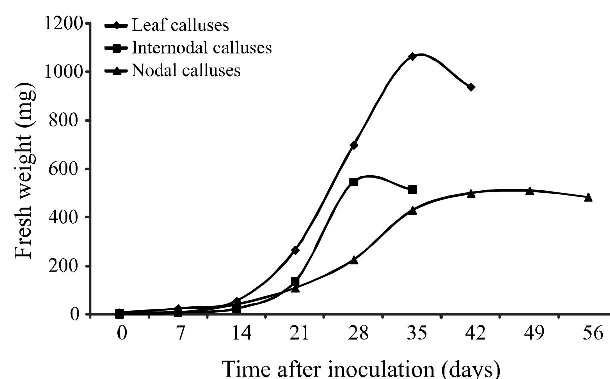
The growth curves of the calluses of the three types of explants followed a sigmoid pattern with six distinct phases; lag, exponential, linear, deceleration, stationary and decline (Figure 1).

The leaf calluses presented a lag phase from the day of inoculation to the 12th day of cultivation, exponential phase from 12th to the 25th, linear from the 25th to the 31st, deceleration from the 31st to the 34th, stationary from the 34th to the 36th, and decline from the 36th to the 42nd day.

On the internodal calluses, the lag phase occurred from the inoculation to the 11th day, exponential phase from 11th to the 22nd, linear from the 22nd to the 26th, deceleration from the 26th to the 29th, stationary from the 29th to the 31st, and decline from the 31st to the 35th day.

The nodal calluses showed a lag phase from the day of inoculation to the 7th day of cultivation, exponential phase from 7th to the 29th, linear from the 29th to the 32nd, deceleration from the 32nd to the 40th, stationary from the 40th to the 50th, and decline from the 50th to the 56th day.

Figure 1 - Growth curve of calluses of Jalapeño plants produced from leaf, nodal and internodal explants



In the scientific literature there were not found studies regarding the determination of callus growth curves for the genus *Capsicum*, and so this is an innovative study. The pattern of the callus curve is dependent on the species and explant under consideration (FEITOSA *et al.*, 2013) and the sigmoid pattern is peculiar to dedifferentiated tissues (PEIXOTO; CRUZ; PEIXOTO, 2011). The growth curves of the three types of explants followed this sigmoid pattern with six phases; lag, exponential, linear, deceleration, stationary and decline. Nogueira *et al.* (2008) identified the same six phases, lasting 120 days. Santos *et al.* (2003) identified only the lag, exponential and linear phases for coffee plants, due to the low growth rate characteristic of the species.

Callus growth curves in general are established to identify the stages or phases of fundamental growth processes, in order to determine the exact moment to subculture the calluses into a new medium (SANTOS; FERREIRA; SARUBO, 2010). These stages are; 1) lag phase: metabolite mobilization starts and synthesis of proteins and specific metabolites occurs, without cell multiplication; 2) exponential phase: cell division reaches the maximum; 3) linear phase: cell division reduces; 4) deceleration phase: cell division decreases and cell expansion occurs - this is when the cells have to be transferred to a new culture medium due to the reduction of nutrients, agar dryness and accumulation of toxic substances; 5) stationary phase: no cell division or weight increase occur, but the secondary metabolites accumulation reaches the maximum; and 6) decline phase: loss of weight due to cellular death (CASTRO *et al.*, 2008; NOGUEIRA *et al.*, 2008; SANTOS; FERREIRA; SARUBO, 2010).

The deceleration phase occurred from the 31st to the 34th day for leaf explants, from the 26th to the 29th for internodal explants, and from the 32nd to the 40th day for

nodal explants. In general the focus of the callus growth curves is the beginning of the deceleration phase, which is the exact moment to subculture the calluses into a new liquid medium in order to establish cell suspensions (SANTOS; FERREIRA; SARUBO, 2010). In this case, the adequate moment to subculture callus cells of Jalapeño plants into a liquid medium is on the 31st, 26th and 32nd day, respectively, for leaf, internodal and nodal explants. Similarly, Balbuena *et al.* (2009) used callus cells of *P. solmsianum* on the 24th day of culture to initiate cell suspension cultures. Santiago (2003) studied the callus growth in *P. hispidinervium* and identified the deceleration phase starting from the 42nd day, from which there was a decrease in the dry mass of the calluses. Valle (2003), studying the callus growth of the same species observed the deceleration phase starting on the 40th day.

CONCLUSIONS

1. For callus induction in *C. annuum* var. *annuum* cv. Iberaba Jalapeño it is recommended to utilize an MS basal culture medium supplemented with 2.22 μM BA + 18.10 μM 2,4-D for leaf and internodal explants and 2.22 μM BA + 9.05 μM 2,4-D for nodal explants, which result in formation of calluses in all the explants with 100% of their area covered by callus cells;
2. The growth curves of these calluses follow a sigmoid pattern, with identifiable lag, exponential, linear, deceleration, stationary and decline phases. The adequate times to subculture callus cells into a new liquid medium in order to establish cell suspensions are on the 31st, 26th and 32nd days of cultivation for leaf, internodal and nodal explants, respectively.

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