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In vitro cultivation of callus cells from nodes and internodes of *Capsicum annuum* cv. All Big

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***Abstract*—** *Cell suspension cultures can be a valuable system for production of secondary metabolites. Capsicum annuum is a pepper species largely studied due to its biologically active compounds. The objective of this study was to establish a protocol for callus induction in nodes and internodes of C. annuum cv. All Big and to determine the callus growth curve, with focus on the deceleration phase, when the callus cells must be cultivated in a liquid medium in order to generate a cell suspension system. Nodal and internodal segments were submitted to media supplemented with the growth regulators 2,4-D and BA in factorial combination. After 49 days, the percentage of explants where callus induction occurred (%CI), the explant area covered by callus cells (ACCC), and the fresh weight of the explants were evaluated. In order to determine the growth curve, the explants were cultivated in the media supplemented with the growth regulators that resulted in the highest callus cell proliferation, weighing the calluses in the subsequent 49 days. The treatments that resulted in the highest %CI, ACCC and callus weight were 4.52 µM 2,4-D without BA for nodal explants and 9.05 µM 2,4-D + 2.22 µM BA for internodal explants. The growth curves of the calluses of the two types of explants followed a sigmoid pattern with six distinct phases; lag, exponential, linear, deceleration, stationary and decline. The deceleration phase started on the 27th and on the 19th day, respectively, for nodal and internodal explants.*
***Keywords*— Callogenesis, growth curve, secondary metabolites.**

I. INTRODUCTION

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).

Capsicum annuum L. is a species of hot pepper which has been largely studied because of its biologically active compounds (Koffi-Nevry et al., 2012). The insecticidal effect of its extract has been demonstrated, causing antifeedant effect in *Spodoptera litura*, a dangerous pest of many economically important crops, and in *Achaea janata*, which attacks leaves of *Ricinus communis* (Devanand and Rani, 2011); its seed powder showed toxic effect against *Sitophilus zeamais* and *Callosobruchus maculatus*, insects that cause damage in stored maize and cowpea, respectively (Oni, 2011). Acaricidal effect were reported against the two-spotted spider mite *Tetranychus urticae*, with high mortality in larva, nymph and adult stages (Erdogan et al., 2010). Its bactericidal or inhibitory effects have been demonstrated against *Streptococcus mutans* (Santos et al., 2012) *Vibrio cholerae*, *Staphylococcus aureus* and *Salmonella typhimurium* (Koffi-Nevry et al., 2012), *Ralstonia solanacearum*, *Clavibacter michiganensis* and *Erwinia carotovora* (Games et al., 2013). Antifungal effects have been reported against *Colletotrichum lindemunthianum*, *Candida tropicalis* (Diz et al., 2011) and *Alternaria solanii* (Games et al., 2013). The identification of the bioactivity of *C. annuum* substances encourages the evaluation of their utilization as alternatives in the control of agricultural pests.

This research is part of a project in which in vitro produced secondary metabolites from Capsicum species are being tested against agricultural pests and diseases. As such, this study provides a protocol for callus induction from nodes and internodes of *C. annuum* cv. All Big and an identification of the callus growth pattern, focusing on the deceleration phase, when the callus cells must be subcultured into liquid medium in order to produce cell suspension cultures and the production of secondary metabolites.

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II. MATERIAL AND METHODS

The experiments were carried out at the Plant Tissue Culture Laboratory at Embrapa (Brazilian Agricultural Research Corporation) in Porto Velho, Brazil. Seeds of *C. annuum* L. cv. All Big 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. Under aseptic conditions, the seeds were individually inoculated into test tubes with 10.0 mL of an MS (Murashige & Skoog, 1962) basal culture medium supplemented with 30.0 g L-1 sucrose and 6.0 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 by cutting the leaves in explants of 1.0 cm2, which were individually inoculated into test tubes with 10.0 mL of an MS basal culture medium as mentioned before, supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) (0, 4.52, 9.05 or 18.10 µM) and 6-Benzylaminopurine (BA) (0, 0.44, 2.22 or 11.10 µM) in factorial combinations. All 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. The treatments were arranged in a completely randomized design. After 49 days, evaluations were done by assessing the percentage of explants where callus induction occurred (%CI); the explant area covered by callus cells (ACCC), according to Mendonça et al. (2013), who established the following scores: 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75% and 4 = 100% of leaf area covered by callus; and the fresh weight of the explants, by using a precision scale. Variance analyses and Tukey tests ($P<0.05$) were performed by using the Assistat 7.5 statistical program.

In order to determine the growth curve, the explants were individually transferred, with the adaxial face up, into test tubes (25 x 150 mm) containing 10.0 mL of an MS basal culture medium as mentioned, supplemented with the growth regulators combination that resulted in the highest callus cell proliferation: 4.52 µM 2,4-D without BA for nodal explants, and 9.05 µM 2,4-D + 2.22 µM BA for internodal explants. The explants were incubated in a growth chamber under the mentioned conditions. In the subsequent 49 days, calluses were carefully separated from the culture medium and weighed. From these data sets the lag, exponential, linear, deceleration and decline phases of callus growth were determined; these data were submitted to regression analysis (Gomes, 2009).

III. RESULTS AND DISCUSSION

In both the nodal and the internodal explants, there was no callus induction on the MS medium without growth regulators, which indicates the necessity of their supplementation for callus formation (Table 1). The absence of callus induction on nodal explants (and low callus induction on internodal explants) was also observed when the highest concentrations of the two growth regulators were used simultaneously - 11.10 µM BA and 18.10 µM 2,4-D, what implies that there is no need to test higher concentrations for callus induction. All the other concentrations of 2,4-D and BA, in combination or not, led to the induction of calluses on the explants. The calluses thereby produced were friable and whitish. As mentioned by Souza et al. (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.

In nodal explants, callus induction in 100% of the explants was observed in the media supplemented with 4.52 µM 2,4-D without BA; 9.05 µM 2,4-D alone or in combination with 0.44, 2.22 or 11.10 µM BA, or 18.10 µM 2,4-D without BA. Different results were obtained for internodal explants, where callus induction in 100% of the explants occurred in the media with 2.22 µM or 11.10 µM BA without 2,4-D; or 4.52 µM 2,4-D without BA; or in the combinations of 9.05 2,4-D with 0.44, 2.22 or 11.10 µM BA. Santos & Smozinski (2017), who studied the proliferation of dedifferentiated cells from internodes of C. annuum cv. Yolo Wonder also found a positive interaction of 2,4-D and BA, but they observed a low efficiency of each of them alone.

Table 1 - Percentages of callus induction (CI), scores for area of the explant covered by callus cells (ACCC) and average fresh weight in nodal and internodal explants of All Big plants submitted to different combinations of BA and 2,4-D in the culture medium, 49 days after inoculation.

2,4-D (µM)	BA (µM)			
	0	0.44	2.22	11.10
	Percentages of CI in nodal explants			
0.0	0 bC*	40 bB	80 aA	20 bBC
4.52	100 aA	80 aB	80 aB	80 aB
9.05	100 aA	100 aA	100 aA	100 aA

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18.10	100 aA	20 bBC	40 bB	0 bC
	Percentages of CI in internodal explants			
0.0	0 bC	40 bB	100 aA	100 aA
4.52	100 aA	80 aB	60 bC	60 bC
9.05	80 aB	100 aA	100 aA	100 aA
18.10	20 bB	40 bA	40 bA	27 cAB
	Scores for ACCC in nodal explants			
0.0	0.0 bB	0.4 cB	3.0 aA	0.2 cB
4.52	4.0 aA	2.0 bC	3.0 aAB	2.4 bC
9.05	3.6 aA	3.8 aA	3.8 aA	3.4 aA
18.10	0.2 bB	1.3 bA	1.2 bA	0.0 cB
	Scores for ACCC in internodal explants			
0.0	0.0 bB	0.6 cB	2.0 bA	2.0 aA
4.52	0.2 bB	2.0 bA	0.6 cB	2.4 aB
9.05	2.8 aB	3.2 aB	4.0 aA	2.8 aB
18.10	2.8 aA	0.4 cB	0.4 cB	0.3 bB
	Average weight (mg) in nodal explants			
0.0	6 cB	22 bB	148 aA	24 bB
4.52	899 aA	328 aB	46 bC	36 bC
9.05	148 bD	404 aA	283 aB	189 aC
18.10	7 cA	11 bA	11 bA	7 bA
	Average weight (mg) in internodal explants			
0.0	2 bB	16 bB	86 bA	11 bB
4.52	3 bB	24 bA	10 bB	18 bAB
9.05	181 aB	333 aB	850 aA	220 aB
18.10	133 aB	8 bB	5 bB	9 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.

In relation to the scores for ACCC, the highest values were obtained with 4.52 µM 2,4-D without BA for nodal explants, and with 9.05 µM 2,4-D + 2.22 µM BA for internodal explants. Confirming these results, the weight of the explantes (and, of course, the calluses formed around it) was highest, for both kinds of explant, in the same concentrations of growth regulators that led to the highest ACCC. Santos & Souza (2016), aiming at the establishment of a protocol for cell suspension from leaves of C. annuumm cv. Etna, recorded 100% callus induction, score 4.0 for explant area covered by callus cells and the highest callus weight by supplementing the media with 4.52 µM 2,4-D + 0.44 µM BA.

The growth curves of the calluses of the two types of explants followed a sigmoid pattern with six distinct phases; lag, exponential, linear, deceleration, stationary and decline (Fig. 1 and 2).

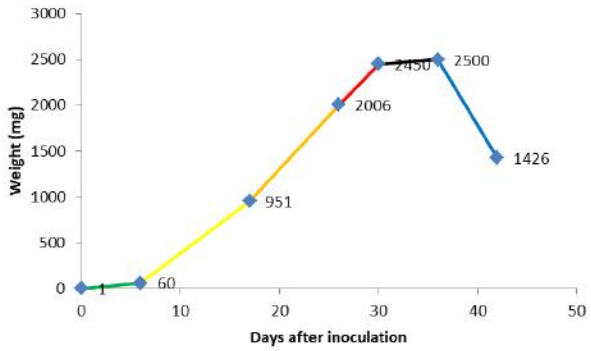

Fig.1: Growth of nodal calluses of C. annuum cv. All Big cultivated in an MS medium supplemented with 4.52 µM 2,4-D, with the lag (green), exponential (yellow), linear (orange), deceleration (red), stationary (black) and decline (blue) phases.

The nodal calluses presented a lag phase from the day of inoculation to the 6th day of cultivation, exponential phase from 7th to the 17th, linear from the 18th to the 26th, deceleration from the 27th to the 30th, stationary from the 31st to the 36th, and decline from the 37th to the 42nd day.

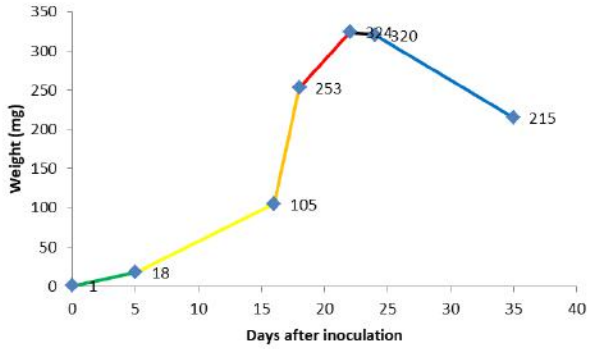

Figure 2 - Growth of internodal calluses of *C. annuum* cv. All Big cultivated in an MS medium supplemented with 9.05 µM 2,4-D + 2.22 µM BA, with the lag (green), exponential (yellow), linear (orange), deceleration (red), stationary (black) and decline (blue) phases.

In the internodal calluses, the lag phase occurred from the inoculation to the 5th day, exponential phase from 6th to the 16th, linear from the 17th to the 18th, deceleration from the 19th to the 22nd, stationary from the 23rd to the 24th, and decline from the 25th to the 35th day.

IV. CONCLUSION

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 et al., 2011). The focus of callus growth curves is to determine the beginning of the deceleration phase, which is the exact moment to

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subculture the calluses into a new liquid medium in order to establish cell suspensions (Santos et al., 2010). In this case, the adequate moment to subculture callus cells from nodal and internodal explants of *C. annuum* cv. All Big into a liquid medium is on the 27th and on the 19th day, respectively.

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