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In vitro cell suspension cultivation systems have been largely reported as safe and standardized methods for production of secondary metabolites with medicinal and agricultural interest. Capsicum annuum is one of the most widely grown vegetable in the world and its biological activities have been demonstrated against insects, fungi, bacteria and other groups of organisms. The determination of procedures for the dedifferentiation of cells into callus cells 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 objective of this study was to establish a protocol for dedifferentiation of leaf cells of the cultivar C. annuum cv. Etna and to determine the growth pattern of the calluses with a focus on the deceleration phase, when the callus cells must be subcultured into a liquid medium in order to establish cell suspension cultivations aiming at the production of secondary metabolites. treatment that resulted in the highest %CI, ACCC and callus weight was the combination of 4.52 µM 2,4-D + 0.44 µM BA. The calluses produced were friable and whitish and their growth pattern followed a sigmoid shape. The deceleration phase started on the 23rd day of cultivation. Callus induction in leaf explants of C. annuum cv. Etna can be achieved in MS medium supplemented with 4.52 µM 2,4-D + 0.44 µM BA, which results in high cellular proliferation; in order to start a cell suspension culture, callus cells on the 23rd day of culture should be used.

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Abstract

In vitro cell suspension cultivation systems have been largely reported as safe and standardized methods for production of secondary metabolites with medicinal and agricultural interest. Capsicum annuum is one of the most widely grown vegetable in the world and its biological activities have been demonstrated against insects, fungi, bacteria and other groups of organisms. The determination of procedures for the dedifferentiation of cells into callus cells 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 objective of this study was to establish a protocol for dedifferentiation of leaf cells of the cultivar C. annuum cv. Etna and to determine the growth pattern of the calluses with a focus on the deceleration phase, when the callus cells must be subcultured into a liquid medium in order to establish cell suspension cultivations aiming at the production of secondary metabolites. treatment that resulted in the highest %CI, ACCC and callus weight was the combination of 4.52 µM 2,4-D + 0.44 µM BA. The calluses produced were friable and whitish and their growth pattern followed a sigmoid shape. The deceleration phase started on the 23rd day of cultivation. Callus induction in leaf explants of C. annuum cv. Etna can be achieved in MS medium supplemented with 4.52 µM 2,4-D + 0.44 µM BA, which results in high cellular proliferation; in order to start a cell suspension culture, callus cells on the 23rd day of culture should be used.

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Introduction

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).

Cell suspension cultivation systems are used for large scale culturing of plant cells from which secondary metabolites are extracted. The advantages of this method are: cell suspensions provide a continuous, reliable source of natural products, which can be produced at a rate similar or superior to that of intact plants (Vanisree et al., 2004); *in vitro* production overcomes the secondary metabolites asymmetrical distribution in the intact plants (Far & Taie, 2009); cultured cells are free of microbes and insects and the cells of any plant can easily be multiplied to yield their specific metabolites (Parsaeimehr & Mousavi, 2009); the production of secondary compounds under controlled conditions prevents fluctuations in concentrations due to geographical, seasonal, and environmental variations (Murthy et al., 2014); and ensures sustainable conservation and rational utilization of biodiversity (Coste et al., 2011). The determination of procedures for the dedifferentiation of cells into callus cells and the subsequent study of the callus growth pattern are necessary for the establishment of cell suspensions (Santos et al., 2015). Protocols for callus induction have been established for some *Capsicum* species and varieties; Kintzios et al. (2000) promoted callus induction in leaf explants of *C. annuum* cv. Colombo, Kittipongpatana et al. (2007) induced callus in leaf explants of *C. annuum*, Umamaheswari and Lalitha (2007) induced formation of large and friable calluses in leaf explants of *C. annuum*, Khan et al. (2011) promoted callogenesis on internodal explants of *C. annuum* cv. Pusa Jwala.

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* L. cv. Etna 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.

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 *Capsicum annuum* L. cv. Etna 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 and 18.10 µM) and 6-Benzylaminopurine (BA) (0, 0.44, 2.22 and 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. 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 + 0.44 µM BA. 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).

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 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.

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 Etna 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 bB*	100 aA	100 aA	20 bB
4.52	100 aA	53 bB	60 bB	60 aB
9.05	100 aA	20 cB	20 cB	20 bB
18.10	100 aA	20 cB	20 cB	0 bB
	Percentages of CI in internodal explants			
0.0	0 bC	67 aB	100 aA	80 aB
4.52	100 aA	40 bB	20 bB	20 cB
9.05	100 aA	40 bB	40 bB	40 bB
18.10	20 bC	40 bB	100 aA	0 cC
	Scores for ACCC in nodal explants			
0.0	0.00 cC	3.40 aA	2.80 aB	0.20 cC
4.52	4.00 aA	1.40 bB	0.60 bD	1.00 aC
9.05	2.80 bA	0.20 cC	0.20 cC	0.80 bB
18.10	4.00 aA	0.20 cB	0.20 cB	0.00 cC
	Scores for ACCC in internodal explants			
0.0	0.00 bD	0.67 aC	2.80 aA	1.40 aB
4.52	4.00 aA	0.80 aB	0.40 cC	0.20 cC

9.05	3.80 aA	0.40 bB	0.40 cB	0.40 bB
18.10	0.20 bC	0.40 bB	1.60 bA	0.00 cC
	Average fresh weight (mg) in nodal explants			
0.0	12.15 dD	94.15 aB	28.18 aC	101.57 aA
4.52	413.88 aA	25.78 bB	11.20 cD	15.64 bC
9.05	62.38 cA	0.47 dD	5.90 dC	14.06 bB
18.10	249.82 bA	3.03 cC	14.44 bB	10.10 cD
	Average fresh weight (mg) in internodal explants			
0.0	15.26 cC	40.14 aB	73.17 aA	60.12 aA
4.52	146.83 aA	44.80 aB	26.08 bC	13.38 bC
9.05	81.84 bA	16.97 bB	14.20 bB	38.18 aB
18.10	18.30 cA	15.21 bA	26.94 bA	3.30 bA

*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 nodal explants, callus induction in 100% of the explants was observed in the media supplemented with 4.52 µM, 9.05 µM or 18.10 µM 2,4-D without BA; 0.44 µM or 2.22 µM BA without 2,4-D. Similar results were obtained for internodal explants, where callus induction in 100% of the explants occurred in the media with 4.52 µM or 9.05 µM 2,4-D without BA; or 2.22 µM BA without 2,4-D. The use of the regulators in combination did not have a positive effect on this variable. The opposite was observed by Santos & Smozinski (2017), who studied the proliferation of dedifferentiated cells from internodes of *C. annuum* cv. Yolo Wonder and found a positive interaction of 2,4-D and BA, as well as the low efficiency of each of them alone.

In relation to the scores for ACCC, the highest values were obtained with 4.52 µM or 18.10 µM 2,4-D without BA for nodal explants, and with 4.52 µM 2,4-D without BA for internodal explants. At this point, it is clear that the use of BA was not necessary in the induction of callus in nodal or internodal explants of Etna.

Confirming these results, the weight of the explantes (and, of course, the calluses formed around it) was highest, for both kinds of explant, with the supplementation of 4.52 µM 2,4-D in the media. 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 (Figures 1 and 2).

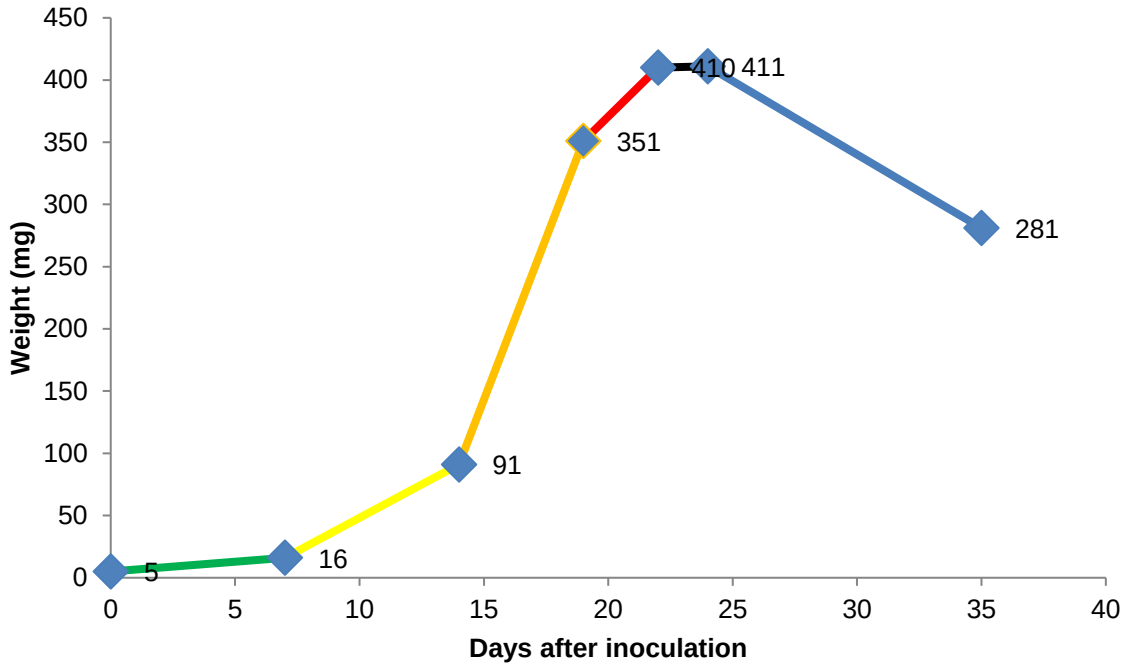

Figure 1 - Growth of nodal calluses of *C. annuum* cv. Etna cultivated in an MS medium supplemented with 4.52 µM 2,4-D, with the lag (green), exponential (yellow), linear (orange), deceleration (red), stationary (black) and decline (blue) phases.

The nodal calluses presented a lag phase from the day of inoculation to the 7th day of cultivation, exponential phase from 8th to the 14th, linear from the 15th to the 19th, deceleration from the 20st to the 22nd, stationary from the 23th to the 24th, and decline from the 25th to the 35th day.

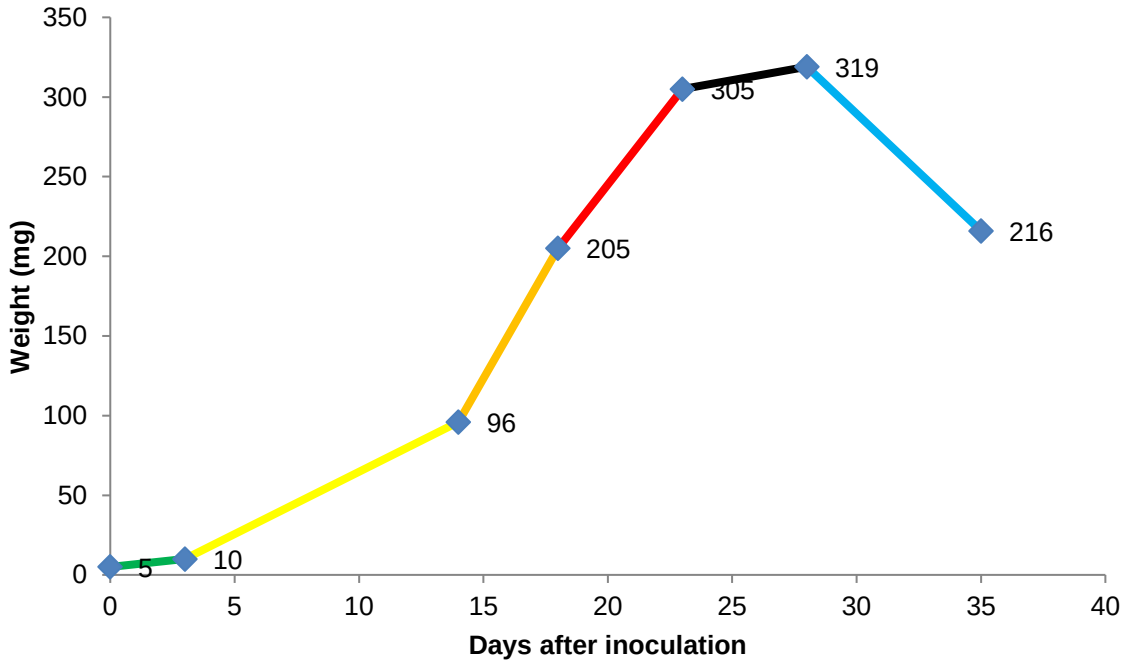

Figure 2 - Growth of internodal calluses of *C. annuum* cv. Etna cultivated in an MS medium supplemented with 4.52 µM 2,4-D, with the lag (green), exponential (yellow), linear (orange), deceleration (red), stationary (black) and decline (blue) phases.

On the internodal calluses, the lag phase occurred from the inoculation to the 3rd day, exponential phase from 4th to the 14th, linear from the 15th to the 18th, deceleration from the 19th to the 23rd, stationary from the 24th to the 28th, and decline from the 29th to the 35th day.

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 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. Etna into a liquid medium is on the 20th and on the 19th day, respectively.

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