AUSTRALIAN JOURNAL OF BASIC AND APPLIED SCIENCES

ISSN:1991-8178 EISSN: 2309-8414
Journal home page: www.ajbasweb.com

Dedifferentiation of Leaf Cells and Growth Pattern of Calluses of *Capsicum annuum* cv. Etna

Mauricio Reginaldo Alves dos Santos and Carolina Augusto de Souza

Embrapa Rondonia, Box.127. Porto Velho. Brazil. 76815-800.

Address For Correspondence:
Mauricio Reginaldo Alves dos Santos, Embrapa Rondonia, Box. 127. Porto Velho. Brazil. 55-69-32195025.
E-mail: mauricio.santos@embrapa.br

ARTICLE INFO

Article history:
Received 28 May 2016
Accepted 29 July 2016
Published 17 August 2016

Keywords:
Callogenesis, growth curve, secondary metabolites.

ABSTRACT

Background: *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. To date, no study has described the development of protocols for callus induction in *C. annuum* L. cv. Etna. **Objective:** 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. **Results:** The 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. **Conclusion:** 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.

INTRODUCTION

Capsicum annuum is a species of hot pepper which has been largely studied because of its biological active compounds (Koffi-Nevry *et al*., 2012). The insecticidal effect of its leaf 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*

To Cite This Article: Mauricio Reginaldo Alves dos Santos and Carolina Augusto de Souza., Dedifferentiation of Leaf Cells and Growth Pattern of Calluses of Capsicum annuum cv. Etna. ***Aust. J. Basic & Appl. Sci.,*** *10(12): 362-368, 2016*

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.

Biotechnological approaches, more specifically plant tissue cultures, have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Rao and Ravishankar, 2002). 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 and 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 and 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 will be tested against agricultural pests and diseases. To date, no study has described the development of protocols for callus induction in *C. annuum* L. cv. Etna. As such, this study provides a protocol for callus induction from leaves 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.

MATERIALS 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 and 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 cm^2, 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

Dedifferentiation became apparent on the 7^{th} day of culture, with a swelling of the explants and from the 7^{th} to the 14^{th} day callus formation could be observed in some 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 small isodiametric cells, rapidly growing, with high frequency of cell division (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.

There was no callus induction on the MS medium without growth regulators, which indicates the necessity of their supplementation for callus formation (Table 1). All the tested combinations of 2,4-D and BA, in combination or not, led to the induction of calluses on the explants. Callus induction in all the explants was observed in five treatments; 11.10 µM BA; 4.52 µM 2,4-D + 0.44 µM BA; 4.52 µM 2,4-D + 2.22 µM BA; 9.05 µM 2,4-D + 0.44 µM BA; and 18.10 µM 2,4-D + 2.22 µMBA. It is possible to establish an optimum range for callus induction, with the combination of 2,4-D from 4.52 to 9.05 µM with BA from 0.44 to 2.22 µM.

Khan *et al*. (2011) studied the effects of 2,4-D and BA on internodal explants of *C. annuum* cv. Pusa Jwala and observed 70% callus induction with the use of 10.0 µM 2,4-D in isolation (in comparison with 60% with 9.05 µM 2,4-D observed in the present study); but the combination of 10.0 µM 2,4-D + 1.78 µM BA resulted in 95% callus induction (in comparison with 60% observed with 9.05 µM 2,4-D + 2.22 µM BA in this study).

Umamaheswari and Lalitha (2007) also recorded the formation of large and friable calluses in leaf explants of *C. annuum*, by supplementing the medium with 9.05 µM 2,4-D in combination with the cytokinin kinetin (KIN) at 2.32 µM. These authors tested several kinds of explants; young leaves, buds, pericarp tissue, nodal segments, and placental region, cultivated in an MS medium with 2,4-D, gibberellic acid (GA_3), indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA) and KIN in diverse combinations. The placental region submitted to the combination of 9.05 µM 2,4-D + 2.32 µM 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.

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 and BA in isolation resulted in axillary bud formation. However, BA at the concentration of 2.22 µM in isolation promoted moderate callus formation in the three types of explants (in the present work this concentration resulted in 40% callus induction in leaf explants).

Callus induction is supposed to be reached with a hormonal balance guaranteed by combinations of exogenous growth regulators; auxins, cytokinins and eventually gibberellins (Santos *et al*., 2015). In general, cytokinins and auxins, or only one of these classes of growth regulators, can be enough to promote the induction; 2,4-D is the most often used auxin for this purpose and has been referred to as essential in some cases (Santos *et al*., 2014a). The auxins are able to start cell division and to control the processes of growth and cell elongation (Nogueira *et al*., 2008). Often, 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*., 2014b).

The ACCC and the weight of the explants followed the same pattern described by the %CI, with a trend of higher callus cell proliferation in a maximum range with combinations of 2,4-D from 4.52 to 9.05 µM with BA from 0.44 to 2.22 µM (Tables 2 and 3).

Specifically in relation to the ACCC, two treatments distinguished themselves from other treatments; 4.52 µM 2,4-D + 0.44 µM BA and 9.05 µM 2,4-D + 0.44 µM BA, with scores of 4.00 and 3.60, respectively. A similar study was developed by Santos *et al*. (2014a), who found the highest ACCC (100%) in *Kalanchoe pinnata* Lam. leaf explants by supplementing the medium with 4.52 µM 2,4-D + 8.88 µM BA, with 91% callus induction and 50 to 100% leaf explants covered by callus cells. Santos *et al*. (2015) evaluated the proliferation of callus cells in leaf explants of *P. carniconnectivum* C. DC. and estimated the maximum ACCC from 47.79 to 48.59%, corresponding respectively to the supplementation of the media with 10.58 µM BA and 9.57 µM 2,4-D. Cerqueira *et al*. (2002) achieved high callus induction in leaf explants of *Tridax procumbens* Linn. with 10.74 µM NAA + 8.88 µM BA, observing 100% of the explant area covered by callus cells.

Evaluating simultaneously the three variables; %CI, ACCC and weight of the explants, the highest values, at a significant level, were observed with the combination of 4.52 µM 2,4-D + 0.44 µM BA, which resulted in 100% callus induction, the score 4.0 (100% of the explant area covered by callus cells) and calluses with an average weight of 713 mg. The same result was observed by Kittipongpatana *et al*. (2007), who found the highest callus cell proliferation in leaf explants of *C. annuum* with the combination of 4.52 µM 2,4-D + 0.44 µM BA. However, the authors do not mention the cultivar or variety utilized. Kintzios *et al*. (2000) used much higher concentrations; 13.6 µM 2,4-D + 9.0 µM BA to promote callus induction in leaf explants of *C. annuum* cv. Colombo.

Almost the same ratio observed in the present study between 2,4-D and BA (1 : 0.1) was found by Nuñez-Palenius and Ochoa-Alejo (2005), who established cell suspension of *C. annuum* var. Tampiqueño 74 by supplementing the MS medium with 6.25 µM 2,4-D + 0.66 µM BA.

Kang *et al*. (2005) induced calluses in *C. annuum* L. cv. P1482 with 9.05 µM 2,4-D, in order to establish a cell suspension culture. In the present study this concentration was not very efficient to promote callus cell proliferation in relation to other treatments. It can be attributed to the type of explant, for the cited authors do not mentioned the organ used as a source of explants. Using roots as a source of explants, Ma (2008) induced calluses in *C. annuum* with a combination of 4.52 µM 2,4-D + 0.46 µM KIN. The same combination and concentrations were used by Islek *et al*. (2014) to produce calluses from *C. annuum* hypocotyl explants. Kehie *et al*. (2012) also used a combination of 2,4-D (9.05 µM) and KIN (2.32 µM) to promote callus induction in hypocotyl tissue of *C. chinense*.

Callus growth. The callus growth pattern followed a sigmoid shape (Figure 1). It was possible to identify a lag phase from the day of inoculation until the 9th day; an exponential phase from the 9th to the 17th day; a linear phase from the 17th to the 23rd day; a deceleration phase from the 23rd to the 29th day; a stationary phase from the 29th to the 33rd day; and a decline phase from the 33rd to the 35th day.

In the scientific literature there were not found studies regarding the determination of callus growth curves for the genus *Capsicum*. 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 *et al*., 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: neither cell division nor 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 *et al*., 2010).

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 leaf explants of *C. annuum* cv. Etna into a liquid medium is on the 23rd day. Likewise, Balbuena *et al*. (2009) used callus cells of *P. solmsianum* on the 24th day of culture to initiate cell suspension cultures. Santos *et al*. (2010) observed the deceleration phase starting on the 43rd day in *C. canephora* calluses; Nogueira *et al*. (2008) identified this phase starting on the 60th day for *Byrsonima intermedia* A. Juss.; and Castro *et al*. (2008) found the beginning of this phase on the 71st day for *Stryphnodendron adstringens* (Mart.) Coville. Kehie *et al*. (2012) mention the callus subculture every 30 days in order to establish cell suspension cultures from hypocotyl tissue of *C. chinense* cv. Naga King Chili.

The determination of a protocol for cellular dedifferentiation and the study of the callus growth pattern in *C. annuum* cv. Etna will allow the establishment of a cell suspension culture. This suspension will provide substances to be tested against agricultural pests and diseases. Furthermore, as observed by Danelutte *et al*. (2005), cell suspension cultures can produce substances that are not produced by the plant that originated it, so they can be a source of new substances and thus new bioactive compounds.

Table 1: Percentages of callus induction in leaf explants of *C. annuum* cv. Etna in an MS medium supplemented with BA and 2,4-D, 49 days after inoculation.

	BA (µM)			
2,4-D (µM)	-	0.44	2.22	11.10
-	0 bB	20 bB	40 bB	100 aA
4.52	40 aB	100 aA	100 aA	60 bB
9.05	60 aB	100 aA	60 bB	13 cC
18.10	40 aB	67 aB	100 aA	60 bB

*Means followed by the same capital letter in the rows or small letter in the columns do not differ significantly at 5% probability by Tukey's test.

Table 2: Scores (from 0.0 to 4.0) for area of the explant covered by callus cells (ACCC) of *C. annuum* cv. Etna leaf explants in an MS medium supplemented with BA and 2,4-D, 49 days after inoculation.

	BA (µM)			
2,4-D (µM)	-	0.44	2.22	11.10
-	0.00 aB	0.40 cB	0.40 bB	2.40 aA
4.52	0.80 aB	4.00 aA	1.40 bB	1.00 bB
9.05	0.80 aB	3.60 aA	0.80 bB	0.40 bB
18.10	0.87 aB	1.73 bA	2.40 aA	1.40 bB

*Means followed by the same capital letter in the rows or small letter in the columns do not differ significantly at 5% probability by Tukey's test.

Table 3: Average fresh weight (mg) of *C. annuum* cv. Etna leaf explants in an MS medium supplemented with BA and 2,4-D, 49 days after inoculation.

	BA (µM)			
2,4-D (µM)	-	0.44	2.22	11.10
-	2.93 aB	33.80 dB	52.25 cB	226.03 aA
4.52	70.93 aD	713.00 aA	252.52 aB	146.62 aC
9.05	112.02 aB	382.41 bA	103.86 cB	28.10 bC
18.10	53.28 aB	144.23 cA	167.44 bA	160.80 aA

*Means followed by the same capital letter in the rows or small letter in the columns do not differ significantly at 5% probability by Tukey's test.

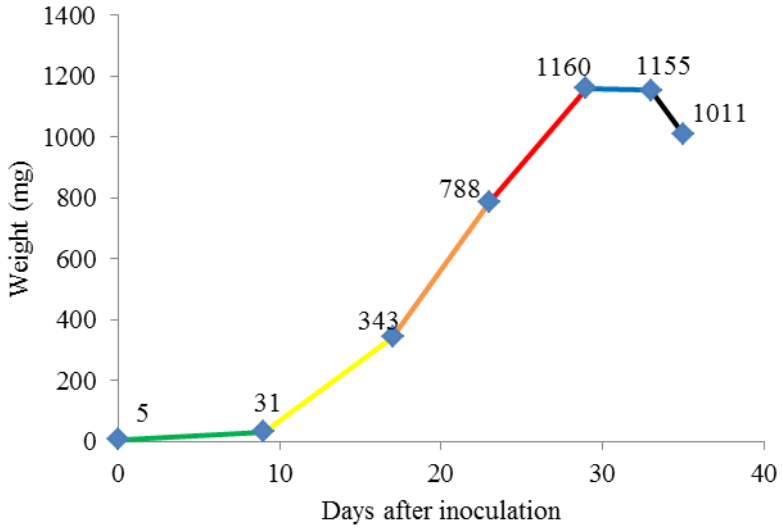

Fig. 1: Growth pattern of *C. annuum* cv. Etna calluses cultivated in an MS medium supplemented with 4.52 µM 2,4-D and 0.44 µM BA, with the lag (green), exponential (yellow), linear (orange), deceleration (red), stationary (blue) and decline (black) phases.

Conclusions:

Callus induction in leaf explants of *C. annuum* cv. Etna can be achieved in MS medium supplemented with 4.52 µM 2,4-D and 0.44 µM BA; callus cells on the 23rd day of culture are appropriate to start a cell suspension culture.

ACKNOWLEDGEMENT

The authors thank EMBRAPA (Brazilian Agricultural Research Corporation) for providing financial support through the scholarship of Souza, C.A.

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