ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 08, Issue, 08, pp. 22080-22084, August, 2018

ORIGINAL RESEARCH ARTICLE OPEN ACCESS

DEDIFFERENTIATION OF LEAF CELLS AND GROWTH OF FRIABLE CALLUSES OF *CAPSICUM ANNUUM* CV. ALL BIG

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ARTICLE INFO

Article History:
Received 27th May, 2018
Received in revised form
18th June, 2018
Accepted 21st July, 2018
Published online 30th August, 2018

Key Words:
Callogenesis,
Growth curve,
Secondarymetabolites.

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 dedifferentiation of leaf cells of the cultivar C. annuumL. cv. All Bigand 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. The explants were inoculated into a medium supplemented with BA and 2,4-D in factorial combinations. The percentage of callus induction (%CI), the explant area covered by callus cells (ACCC) and the weight of the calluses 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 %CI, ACCC andweight were observed with 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 22nd day of cultivation.

Citation: Santos, M.R.A. and Souza. 2018. "Dedifferentiation of leaf cells and growth of friable calluses of capsicum annuum cv. all big", *International Journal of Development Research*, 8, (08), 22080-22084.

INTRODUCTION

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 and Pav. (Bianchetti, 1996). This genus is comprised of sweet and hot peppers (Carvalho *et al*., 2003), which are characterized byhaving high concentrations of antioxidants, vitamins C, E and A, B complex, betacarotene and beta cryptoxanthin (Domenico *et al*., 2012); and mostly its use is related to culinary products. Its fruits are consumed *in natura* or processed as condiments (canned, dyes, additives, etc.) and in the composition of medicines or animal feeds (Barbosa *et al*, 2002; Wagner, 2003). There are studies related to the use of its substances as mutagenic, analgesic, vasodilatory agents and in phytotherapy (Stewart *et al*., 2005). Also, 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).

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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 suspensionculture systems are used for large scale culturing of plant cells from which secondary metabolites are extracted. The advantage of this method is that it can ultimately 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). These cultures offer the possibility of obtaining desirable amounts of compounds as well as ensuring sustainable conservation and rational utilization of biodiversity (Coste *et al*., 2011). Besides, *in vitro* production of secondary compounds under controlled conditions prevents fluctuations in concentrations due to geographical, seasonal, and environmental variations (Murthy *et al*., 2014). 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. 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. All Big. As such, this study provides a protocol for callus induction from leavesand 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

Callus induction: The experiments were carried out at the Plant Tissue Culture Laboratory at Embrapa (Brazilian Agricultural Research Corporation) in Porto Velho, Brazil. Seeds of *Capcisumannuum*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 (Murashigeand 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 18.10µM) and6-Benzylaminopurine(BA) (0, 0.44, 2.22 and 11.10µM) in factorial combinations. The growth regulators, their concentrations and their combinations were tested based on successful studies on callus induction in *Capsicum* species and varieties; *C. annuum* (Kintzios *et al*., 2000; Farias Filho, 2006; Kittipongpatana *et al*., 2007; Umamaheswari and Lalitha, 2007), *C. annuum*cv. PusaJwala (Khan *et al*., 2011), and*C. chinense*(Farias Filho, 2006). All the explants were incubated in a growth chamber at 26±1°C under light provided by cool white fluorescent tubes (50 $\mu molm^{-2}s^{-1}$) 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 inductionoccurred (%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.

Determination of 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 + 0.44µMBA. The explants were incubated in a growth chamber under the mentioned conditions. In the subsequent 49 days, every seven days, three 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 (Pimentel-Gomes, 2009).

RESULTS AND DISCUSSION

Dedifferentiation became apparent on the 7th day of culture, with a swelling of the explants and from the 7thto the 14th 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, and the concentration of 4.52 2,4-D in isolation also did not result in callus formation (Table 1). Except for that, all the tested combinations of 2,4-D and BA, in combination or not, led to the induction of calluses on the explants. The combination of the highest concentrations of the two regulators resulted in the lowest level of callus induction, suggesting that this combination reached a toxic effect. Callus induction in all the explants was observed in eight treatments; 2.22 µM BA, 11.10µM BA, 4.52 µM 2,4-D + 0.44µM BA, 4.52 µM 2,4-D + 2.22 µM BA, 4.52 µM 2,4-D + 11.10 µM BA, 9.05 µM 2,4-D + 0.44 µM BA, 9.05 µM 2,4-D + 2.22µM BA, and 18.10 µM 2,4-D + 2.22µM BA.It is possible to establish an optimum range forcallus induction; with the combination of 2,4-D from 4.52 to 9.05 µM with BA from 0.44 to 2.22 µM.

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

BA (µM)	2,4-D (µM)			
	-	4.52	9.05	18.10
-	0 cC	0 bC	60 cB	80 bA
0.44	87 bB	100 aA	100 aA	80 bC
2.22	100aA	100aA	100aA	100aA
11.10	100aA	100aA	80 bB	20 cC

*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.

Similar results were achieved by Khan *et al*. (2011), who studied the effects of 2,4-D and BA on internodal explants of *C. annuum*cv. PusaJwala and observed 70% callus induction with the use of 10.0 µM2,4-D in isolation (in comparison with 60% with 9.05 µM2,4-D observed in the present study); and in combination with 1.78 µMBA, 95% callus induction (in comparison with 100% observed with 9.05 µM2,4-D + 2.22 µMBA in this study). Also similar were the results reported by Farias Filho (2006), who achieved 80% callus induction in *C. annuum* anthers with 9.05 µM2,4-D and 66% with 6.79µM2,4-D. Umamaheswari and Lalitha (2007) also recorded the formation of large and friable calluses in leaf explants of *C. annuum*, by supplementing the medium with9.05 µM2,4-D in combination with the cytokinin kinetin (KIN) at2.32µM. 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 kinetin (KIN) in diverse combinations. The

placental region submitted to the combination of 9.05 µM2,4-D + 2.32µMKIN 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 threetypes of explants (in the present work this concentration resulted in 100% 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, 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 callogenesis 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). 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 4.52µM 2,4-D and 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 2,376 mg, respectively.

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

BA (µM)	2,4-D (µM)			
	-	4.52	9.05	18.10
-	0.0 dC	0.0 cC	1.2 dA	0.8 bB
0.44	1.1 cC	4.0 aA	1.6 cB	0.8 bD
2.22	1.4 bC	3.8 bA	3.2 aB	1.0 aD
11.10	2.4 aB	3.8 bA	2.4 bB	0.2 cC

*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 weight (mg) of *C. annuum*cv. All Bigleaf explants in an MS medium supplemented with BA and 2,4-D, 49 days after inoculation

BA (µM)	2,4-D (µM)			
	-	4.52	9.05	18.10
-	61 dC	225 cC	937 dA	550 bB
0.44	469 cC	2,376 aA	1,057 cB	375 bD
2.22	920 bC	1,744 bA	1,051 aB	251 aD
11.10	997 aB	1,433 bA	553 bB	88 cC

*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.

The same result was observed by Kittipongpatana *et al*. (2007), who found the highest callus cell proliferation in leaf explants of *C. annuum*withthe combination of 4.52µM 2,4-D + 0.44 µM BA. Much higher concentrations; 13.6µM 2,4-D + 9.0 µM BA, were effective for callogenesis in the same species and explant in the study of Kintzios *et al*. (2000). A similar study was developed by Santos *et al*. (2014a), who found the highest (100%) ACCC in *Kalanchoepinnata*Lam. leaf explants by supplementing the medium with4.52 µM 2,4-D + 8.88 µM BA, with91% 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 47.79to 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 *Tridaxprocumbens* Linn. with10.74 µM NAA + 8.88 µM BA, observing 100% of the explant area covered by callus cells. *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 7th day; an exponential phase from the 7thto the 14th day; a linear phase from the 14th to the 21st day; a deceleration phase from the 21st to the 28th day; and a decline phase from the 28th to the 35th day.

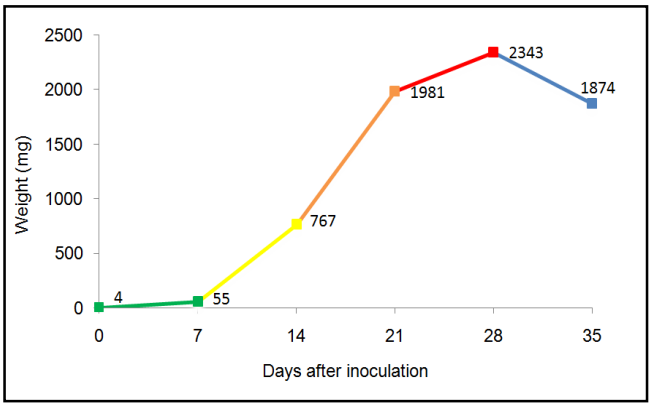

Figure 1. Growth pattern of *C. annuum*cv. All Bigcalluses cultivated in an MS medium supplementedwith 4.52 µM 2,4-D and 0.44 µM BA, with the lag (green), exponential (yellow), linear (orange), deceleration (red), and decline (blue) phases

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. All Big into a liquid medium is on the 21st day. 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. 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 *Stryphnodendronadstringens* (Mart.) Coville.

Conclusion

Callus induction in leaf explants of *C. annuum*cv. All Bigcan be achieved in MS medium supplemented with 4.52 µM 2,4-D and 0.44 µM BA; callus cells on the 21st day of culture are appropriate to start a cell suspension culture.

Acknowledgment

The authors thank Embrapa (Brazilian Agricultural Research Corporation) for providing scholarship of Souza, C. A.

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