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DEDIFFERENTIATION AND GROWTH OF FRIABLE CALLUSES FROM STEMS OF CAPSICUM CHINENSE CV. AIRETAMA

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ABSTRACT

Chili peppers belong to the genus *Capsicum* and have been studied for their bioactivity, provided by secondary metabolites, which can be used for medicinal and agricultural purposes. Some *Capsicum* species have been submitted to in vitro cultivation systems of cell suspensions, which have been largely reported as safe and standardized methods of production of secondary compounds. The objective of this study was to determine a protocol for the establishment of cell suspensions from stems of the cultivar *C. chinense* cv. Airetama. For this, it was necessary to determine the combinations and concentrations of growth regulators for callus induction and to study the growth pattern of the calluses with a focus on the deceleration phase. In this phase the callus cells must be subcultured in a liquid medium in order to establish cell suspension cultivations. Stem explants were inoculated into media supplemented with 2,4-D and BA. The proliferation of callus cells and their growth pattern were evaluated. The highest callus cell proliferation occurred with the combination of 18.10 µM 2,4-D + 2.22 µM BA. The calluses were friable and whitish and their growth pattern followed a sigmoid shape. The deceleration phase started on the 42nd day.

INTRODUCTION

Chili peppers belong to the genus *Capsicum*and are widely grown for their fruits – which may be eaten fresh or cooked, used as a dried powder, or processed into oleoresins. The pungency of the *Capsicum* species is owing to capsaicinoids, nonvolatile alkaloids which are acid amides of C9-C11 branched-chain fatty acids and vanillylamine (Wesolowska *et al*., 2011). There are studies related to the use of its substances as mutagenic, analgesic and vasodilatory agents and in phytotherapy(Stewart*et al*., 2005).Also, alkaloids, flavonoids, polyphenols and sterols have been identified as biological active compounds of *Capsicum* species (Koffi-Nevry*et al*., 2012), which motivates the evaluation of their use as alternatives in the control of agricultural pests. The bioactivity of extracts and isolated substances from plants of this genushas been largely studied. It has been demonstrated that plants of this genus have insecticidal (Erdogan *et al*., 2010; Devanand; Rani, 2011; Oni, 2011), bactericidal (Santos *et al*., 2012; Koffi-Nevry*et al*., 2012; Games *et al*., 2013) and fungicidal activities (Diz *et al*., 2011; Games *et al*., 2013).

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The species C. chinense Jacq. has become increasingly important as a result of its wide diversity and high fruit pungency, which makes it very desirable in many countries (Medina-Lara *et al*., 2008). It was originally found in the Amazon basin, but is commercially grown throughout southern and northern Brazil, due to its adaptability to different soil and local climates. Fruits from this species show an enormous variability in size and shape, and in different intensities of yellow, orange or red when ripe. The pungency of the fruits is due to the synthesis and accumulation of the alkaloid cap saisin in the placental tissue and is one of the most important aspects considered by the spice industry (Maillard*et al*., 1997; Bosland andVotava, 1999; Lanne*et al*., 2007). The high content of phytochemical substances and their functional properties – antioxidant activity, inhibition of α-amylase and butyrylcholinesterase – suggests the utilization of *C. chinense*in nutraceutical products (Menichini *et al*., 2009). Biotechnological approaches – more specifically plant tissue cultures – have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Rao andRavishankar, 2002). Cell suspensioncultivation 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 adequate amounts of compounds as well as ensuring sustainability and rational utilization of biodiversity (Coste *et al*., 2011).Besides, *in vitro* production of secondary compoundsunder 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. To date, no study has described protocols for callus induction in *C. chinense*cv. Airetama. The development of procedures for thededifferentiation 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 growth regulators, their concentrations and their combinations were tested in this study based on successful studies on callus induction in *Capsicum* species and varieties(Kintzios *et al*., 2000; Kittipongpatana *et al*., 2007; Umamaheswari andLalitha, 2007; Khan *et al*., 2011; Santos and Souza, 2016).

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 *Capsicumchinense*Jacq.cv.Airetama 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 which contained 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, and 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 stems in explants of 1.0 cm length, 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 11.10µM) 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 $\mu molm^{-2}s^{-1}$), 16 hours a day. Treatments were arranged in a completely randomized design with three replications per treatment, each one composed by three explants. After 49 days, evaluations were done by assessing the percentage of callus induction (%CI), the explant area covered by callus cells (ACCC) and weight of the explants. The ACCC was evaluated according to Mendonça*et al*. (2013),who established the following scores: 0 = 0%, 1 = 25%, 2 = 50%, 3 = 75% and 4 = 100% of explant area covered by callus. The weight of the explants was obtained 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 stem explants were individually transferred 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; 18.10 µM 2,4-D + 2.22 µM BA.The explants were incubated in a growth chamber under the mentioned conditions. In the subsequent 63 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

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. There was callus induction in all the explants inoculated on the MS medium without growth regulators (Table 1).

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

2,4-D (µM)	BA (µM)			
	-	0.44	2.22	11.10
-	100aA	67bB	56cC	100aA
4.52	100aA	100aA	100aA	100aA
9.05	100aA	100aA	100aA	100aA
18.10	100aA	100aA	100aA	100aA

*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. chinense*cv. Airetamastem explants in an MS medium supplemented with BA and 2,4-D, 49 days after inoculation

2,4-D (µM)	BA (µM)			
	-	0.44	2.22	11.10
-	1.00aA	1.00aA	1.00aA	1.00aA
4.52	4.00aA	4.00aA	4.00aA	3.78aA
9.05	4.00aA	4.00aA	4.00aA	3.44aA
18.10	4.00aA	4.00aA	4.00aA	4.00aA

*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. chinense*cv. Airetamastem explants in an MS medium supplemented with BA and 2,4-D, 49 days after inoculation

2,4-D (µM)	BA (µM)			
	-	0.44	2.22	11.10
-	0.0561cA	0.0277cA	0.0212dA	0.0098bA
4.52	1.2555bA	1.3169bA	0.6649cB	0.1032bC
9.05	1.7430aAB	1.9580aA	1.4791bB	0.0859bC
18.10	1.8518aAB	1.6961abB	2.1600aA	1.2088aC

*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.

However, this induction was not followed by a significant proliferation of callus cells. The only treatment that did not result in callus induction was 11.10 µM BA without 2,4-D. It was also observed that the lowest percentages of induction occurred when BA was used alone.Callus induction in all the explants was observed in all treatments supplemented with BA and 2,4-D. Used alone, 2,4-D also resulted in 100% of callus induction. The ACCC also was lower when BA was used alone or at the highest concentration (11.10 µM) (Table 2). In the other treatments, all explants were covered by callus cells (100% ACCC), even when 2,4-D was used alone.

In general, it is possible to infer that the supplementation of BA in the medium had a negative effect for callus induction in *C. chinense*cv. Airetama. Evaluating simultaneously the three variables; %CI, ACCC and weight of the explants (Table 3), the highest values, at a significant level, were observed with the combination of 18.10 µM 2,4-D +2.22 µM BA, which resulted in 100% callus induction, the maximum score of 4.0 for the explant area covered by callus cells,and calluses with an average weight of 2,160 mg. *Callus growth.* The callus growth pattern followed a sigmoid shape (Figure 1).

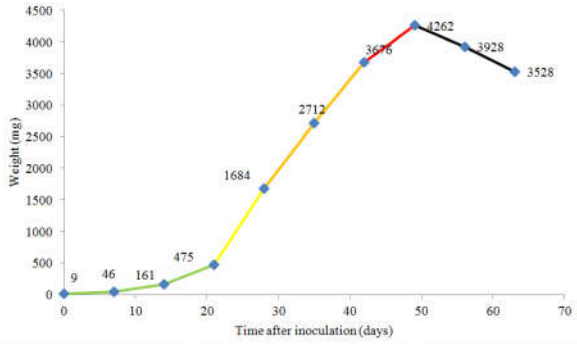

Figure 1. Growth pattern of *C. chinense*cv. Airetamastem calluses cultivated in an MS medium supplemented with 18.10 µM 2,4-D and 2.22 µM BA, with the lag (green), exponential (yellow), linear (orange), deceleration (red) and decline (black) phases

It was possible to identify a lag phase from the day of inoculation until the 21st day; an exponential phase from the 21stto the 28th day; a linear phase from the 28th to the 42nd day; a deceleration phase from the 42nd to the 49th day; and a decline phase from the 49thto the 63rd day. It is possible to establish thatthe adequate moment to subculture callus cells from stem explants of *C. chinense*cv. Airetama into a liquid medium is on the 42ndday.

DISCUSSION

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. The absence of callus in the treatment with 11.10 µM BA (without 2,4-D) probably is due to the excess of cytokinins (endogenous and exogenous) in relation to auxins.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).Khan *et al*. (2011) studied the effects of 2,4-D and BA on internodal explants of *C. annuum*cv. PusaJwala. The highest percentage (95%) of callus induction was reached with 10.0 µM2,4-D and 2.0 µMBA; in comparison with 100% with 9.05 µM2,4-D + 2.22 µMBA observed in the present study. In general, the supplementation of BA had a negative effect for callus induction in the present study. Umamaheswari andLalitha (2007) also mentioned that generally high concentrations of auxins and low concentrations of cytokinins in the medium promote abundant cell proliferation with formation of callus.

The authors recorded the proliferation of callus cells in stem explants of *C. annuum*, by supplementing the medium with 2,4-D in combination with the auxins 1-naphthaleneacetic acid (ANA) and indole-3-acetic acid (IAA); 4.52 µM 2,4-D + 2.69 µM NAA, 4.52 µM 2,4-D + 5.37 µM NAA, 2.26 µM 2,4-D + 2.69 µM NAA, and 4.52 µM 2,4-D + 2.85 µM IAA. 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), (IAA), and Kinetin (KIN) in diverse combinations. The placental region submitted to the combination of 9.05 µM2,4-D + 2.32 µMKIN surpassed all the other treatments in relation to the production of callus and this tissue is being used for the commercial production of capsaicin.

Different results were achieved by Santos *et al*. (2014a), who found the highest ACCC (100%) in *Kalanchoepinnata*Lam. leaf explants by supplementing the medium with 4.52 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 47.79 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 *Tridaxprocumbens* Linn. with 10.74 µM NAA + 8.88 µM BA, observing 100% of the explant area covered by callus cells. 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 56% callus induction in stem explants. 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). Almost the same ratio between 2,4-D and BA (1 : 0.1) observed in the present study for callus induction and proliferation of callus cells was found by several authors. Kittipongpatana *et al*. (2007), achieved 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; Nuñez-Palenius and Ochoa-Alejo (2005), 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; 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; this same combination was 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*. 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. 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 the 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.

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 study was established that the 42nd day after inoculation is the adequate moment to subculture callus cells from stem explants into a liquid medium. Likewise, Santos *et al*. (2010) observed the deceleration phase starting on the 43rd day in *Coffeacanephora*calluses. Differently, Santos and Souza (2016) observed the start of the deceleration phase on the 23rd day of cultivation of leaf explants of *C. annuum*cv. Etna. Balbuena *et al*. (2009) used callus cells of *P. solmsianum* on the 24th day of culture to initiate cell suspension cultures. Nogueira *et al*. (2008) identified the deceleration 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. 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.

Conclusion

Callus induction in stem explants of *C. chinense*cv. Airetama can be achieved in MS medium supplemented with 18.10 µM 2,4-D and 2.22 µM BA. Callus cells on the 42nd day of culture are appropriate to start a cell suspension culture.

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