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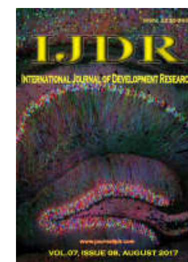

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EVALUATION OF THE PROLIFERATION OF DEDIFFERENTIATED CELLS OF YELLOW BELL PEPPER (*CAPSICUM ANNUUM* CV. PIMENTÃO AMARELO)

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ABSTRACT

Capsicum annuum is notable for its secondary metabolites, which can be of great interest for use in agriculture as alternatives to agrochemicals. Cell suspensions have been used for *in vitro* large-scale production of secondary metabolites. The aim of this study was to dedifferentiate leaf cells of *Capsicum annuum* cv. Pimentão Amarelo (yellow bell pepper) into calluses, and to study their growth pattern, focusing on the deceleration phase, when they can be subcultured to establish cell suspensions. Leaf explants were inoculated on MS medium supplemented with the growth regulators 2,4-D (0, 4.52, 9.05 and 18.10 µM) and BA (0, 4.44, 8.88 and 17.76 µM) in factorial combinations. Callus formation was evaluated weekly for 42 days, by assessing the number of callus induced per treatment and the weight of the explants. The culture medium supplemented with 4.52 µM 2,4-D and 4.44 µM BA resulted in the highest callus induction and weight of callus. For the identification of the growth curve the explants were submitted to this combination and in the subsequent 42 days, every seven days, the weight of the calluses was periodically measured. The growth curve has a sigmoid pattern and the deceleration phase begins 21 days after inoculation.

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INTRODUCTION

It is well established that secondary metabolites play an important role in plant chemical defense (Morais *et al*., 2007), besides those attractants that promote pollination and seed dispersal (Vanin *et al*., 2008). In basic terms, the plants all share a similar biochemistry necessary for a living cell, but in addition to that they produce a wide variety of secondary metabolites, which are involved in interactions between organisms. Considering the number of organisms, and the almost infinite number of interactions possible, it is not surprising that an enormously wide variety of secondary metabolites has evolved within organisms (Veerporte, 1998). Biotechnological approaches, more specifically plant tissue cultures, have potential as a supplement to traditional agriculture in the industrial production of secondary metabolites (Rao & Ravishankar, 2002).

Cell suspension culture systems are used for large scale culturing of plant cells from which bioactive plant 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). The first step to iniciate these culture systems is to establish procedures for the dedifferentiation of specialized plant cells into meristematic and unspecialized callus cells (Santos and Souza, 2016). Calluses are aggregates of unorganized cells, a coherent and amorphous tissue formed

when plant cells multiply in a disorganized way, under the stimulus of growth regulators. During this process, cell differentiation and specialization, which may have been occurring in the intact plant, are reversed, giving rise to a tissue composed of meristematic and unspecialized cell types (George, 2008). In the establishment of cell suspensions, it is also important to evaluate the growth pattern of the calluses of dedifferentiated cells, aiming at the identification of its deceleration phase, when the calluses must be subcultured into a liquid medium, where their cells are disaggregated and cultivated under agitation (Santos *et al*., 2015). The genus *Capsicum* has been studied due to the abundance of secondary metabolites of agricultural interest as alternatives in the control of parasites such as flavonoids, cumarins, saponins and essential oils (Luz, 2007). 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 study describes the dedifferentiation of specialized leaf cells of *Capsicum annuum* cv. Pimentão Amarelo (yellow bell pepper) and analyses the growth of aggregates of dedifferentiated cells as a contribution to the exploration of the potential of these cells in the *in vitro* production of secondary metabolites of relevant importance to agriculture.

MATERIALS AND METHODS

The experiments were carried out at the Plant Tissue Culture Laboratory at Embrapa (Brazilian Agricultural Research Corporation). Seeds of *C. annuum* cv. Pimentão Amarelo 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. In an aseptic hood, the seeds were individually inoculated into test tubes with 10 mL of an MS (Murashige and Skoog, 1962) basal culture medium supplemented with 30 g L^{-1} sucrose and 6 g L^{-1} agar, pH 5.8, autoclaved at 121 °C for 20 minutes. After 42 days of cultivation, the plants were approximately 8 cm tall. Under aseptic conditions, the leaves were cut in explants of 1.0 cm^2 and inoculated individually into test tubes with 10 mL of an MS basal culture medium containing 30 g L^{-1} sucrose and 6 g L^{-1} agar, pH 5.8, autoclaved at 121°C for 20 minutes. The medium was supplemented with benzylaminopurine (BA) (0, 4.44, 8.88 and 17.76 µM) and 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 4.52, 9.05 and 18.10 µM) in a factorial combination. 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. Callus formation was evaluated weekly until the 42nd day, by assessing the number of callus induced per treatment and the fresh weight of the explants. Variance analyses and Tukey test ($P<0.05$) were performed by using the Assistat 7.5 statistical program. For the identification of the growth curve the explants were individually inoculated into test tubes containing an MS basal culture medium as described, supplemented with the growth regulators combination that resulted in the highest callus cell proliferation; 4.44 µM 2,4-D + 4.52 µM BA. The explants were incubated at the described conditions. In the subsequent 42 days, every seven days, three calluses from each treatment were carefully separated from the culture medium and weighed on a precision scale in order to determine their fresh weight. From these data sets the lag, exponential, linear, deceleration, stationary and decline phases of callus growth were determined. These data were submitted to regression analysis.

RESULTS

Seven days after inoculation in media supplemented with growth regulators, there was explant swelling and the consequent onset of callus formation. After 42 days, the calluses, where they occurred, were friable and whitish. In the medium without growth regulators, callus induction was not observed, demonstrating the need of their supplementation for induction (Table 1).

Table 1. Percentages of callus induction in *C. annuum* cv. Pimentão Amarelo leaf explantes in an MS medium supplemented with 2,4-D and BA, 42 days after inoculation

	2,4-D (µM)			
BA (µM)	0	4.52	9.05	17.10
0	0 Bc	20 Bab	30 Ba	30 Ca
4.44	0 Bc	100 Aa	100 Aa	50 ABb
8.88	40 Ac	100 Aa	100 Aa	60 Ab
17.76	40 Ab	100 Aa	100 Aa	40 BCb

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

Table 2. Average weight (mg) of *C. annuum* cv. Pimentão Amarelo leaf explantes in an MS medium supplemented with 2,4-D and BA, 42 days after inoculation

	2,4-D (µM)			
BA (µM)	0	4.52	9.05	17.10
0	129 Bc	325 Cb	442 Ca	533 Ca
4.44	124 Bd	2,525 Aa	2,098 ABb	1,150 Ac
8.88	782 Ac	2,037 Ba	1,998 Ba	1,249 Ab
17.76	813 Ad	2,025 Bb	2,128 Aa	914 Bc

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

Treatment with 4.44 µM BA also did not result in callus induction in the explants. The positive interaction between 2,4-D and BA, as well as the low efficiency of each of them alone, is evident. It is possible to observe an optimum induction range (100%), with the supplementation of the culture medium with 4.52 and 9.05 µM 2,4-D in combination with 4.44, 8.88 and 17.76 µM BA. It can be inferred that, above 9.05 µM 2,4-D, there was saturation, or toxic effect of the growth regulator on the explants. The same pattern observed for callus induction percentages was repeated for callus cell proliferation, evaluated by the weight of the explants. The highest values observed were treatments with combinations of 4.52 and 9.05 µM 2,4-D and 4.44, 8.88 and 17.76 µM BA (Table 2). The treatment with 4.52 µM 2,4-D and 4.44 µM BA is highlighted, showing the maximum cell proliferation, with an average of 2,125 mg per explant. The growth curve of the calluses showed the lag phase from the day of inoculation until the 2nd day (Figure 1); the exponential phase was observed between the 2nd and the 16th day; the linear phase occurred between the 16th and the 20th day; the deceleration phase between the 20th and the 32nd day; the stationary phase between the 32nd and the 34th day; and the declining phase from the 34th to the 42nd day after inoculation.

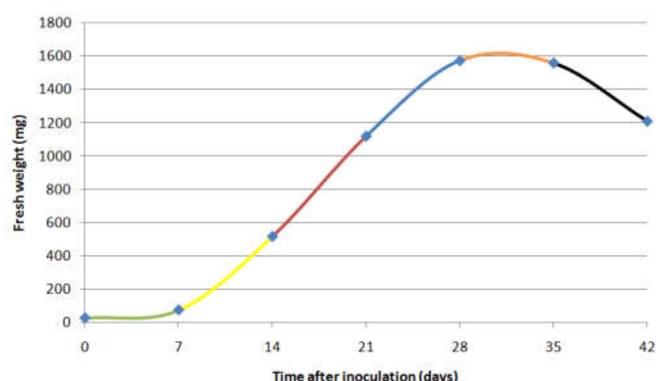

Figure 1. Growth pattern of *C. annuum* cv. Pimentão Amarelo leaf calluses cultivated in an MS medium supplemented with 4.52 µM 2,4-D and 4.44 µM BA, with the lag (green), exponential (yellow), linear (orange), deceleration (blue), stationary (orange), and decline (black) phases

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 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 (Santos *et al*., 2017). In general an adequate balance between auxins and cytokinins is needed to the dedifferentiation of specialized cells. The use of 2,4-D and BA has been suggested for several species or cultivars of *Capsicum*. Santos and Souza (2016) promoted formation and proliferation of callus cells from leaf explants of *C. annuum* cv. Etna by supplementing the medium with a combination of 4.52 µM 2,4-D and 0.44 µM BA. Santos *et al*. (2017) also promoted the proliferation of callus cells from leaf explants of *C. annuum* cv. Jalapeño with 18.10 µM 2,4-D in combination with 2.22 µM BA. Khan *et al*. (2007) induced friable calluses in internodes of *C. annuum* cv. Pusa Jwala with a combination of 9.99 µM 2,4-D and 1.78 µM BA. Kittipongpatana *et al*. (2007) obtained friable calluses from leaf explants of *C. annuum* with a combination of 4.52 µM 2,4-D and 0.44 µM BA. Similar concentrations to those found in the present study were described by Kintzios *et al*. (1996), who induced calluses in leaf explants of *C. annuum* with 12.9 µM BA and 13.6 µM 2,4-D.

Other studies on the callogenesis in *C. annuum* explants report the use of 2,4-D in combination with the cytokinin KIN (Agrawal *et al*., 1989; Andrijany *et al*., 1999; Umamaheswari and Lalitha, 2007), or even with 2,4-D alone (Farias Filho, 2006; Kittipongpatana *et al*., 2007). 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 growth curves of the three types of explants followed this sigmoid pattern with six phases; lag, exponential, linear, deceleration, stationary and decline. Nogueira *et al*. (2008) identified the same six phases, lasting 120 days. Santos *et al*. (2003) identified only the lag, exponential and linear phases for coffee calluses, due to the low growth rate characteristic of the species. 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. 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: no cell division or 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). In general the focus of the callus growth curves is 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 of *C. annuum* cv. Pimentão Amarelo into a liquid medium is on the 20th day.

Conclusion

Callus induction in leaf explants of *C. annuum* cv. Pimentão Amarelo can be achieved in MS medium supplemented with 4.52 and 9.05 µM 2,4-D in combination with 4.44, 8.88 and 17.76 µM BA. Callus cells on the 20th day of culture are adequate to start a cell suspension culture.

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