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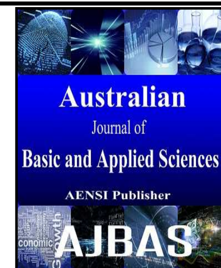

Proliferation Of Dedifferentiated Cells From Internodes Of Bell Pepper(*capsicum annuum* cv. Yolo wonder)

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ABSTRACT

Background: Secondary metabolites are compounds produced by plants in order to protect themselves from attack of herbivores or to attract pollinators or seed dispersers. These substances have been used in agriculture as alternatives to agrochemicals. Methods of tissue culture can be used to promote the *in vitro* production of secondary metabolites from cells, which are kept in liquid culture medium under agitation – what is called a cell suspension system.The first step to establish these culture systems is the determination of procedures for the dedifferentiation of specialized plant cells into callus cells.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. **Objective:** The aim of this study was to dedifferentiate cells of internodes of *Capsicum annuum* cv. Yolo Wonder into calluses, supplementing the culture medium with the growth regulators 2,4-D (0, 4.52, 9.05 and 18.10 µM) and BA (0, 0.44, 2.22 and 11.10 µM) in factorial combinations; and to evaluate the proliferation of callus cells in the different combinations of regulators. **Results:** The culture medium supplemented with 4.52 µM 2,4-D and 2.22 µM BA resulted in the highest callus induction and proliferation. This combination of growth regulators induced callus in all the explants, which were totally covered by callus cells 42 days after inoculation, with the highest average weight of 2,374 mg per explant. **Conclusion:** *In vitro* induction and proliferation of callus in internodes of *C. annuum* cv. Yolo Wonder can be achieved in MS medium supplemented with 4.52 µM 2,4-D in combination with 2.22 µM BA.

INTRODUCTION

One of the main agricultural problems relates to the control of pests and diseases. The use of chemical insecticides compromises the quality of food, causes cumulative effects on the environment and favors the development of resistance in insects and other organisms. These aspects have encouraged studies on new control techniques, which include the use of natural products that are less aggressive to the environment (Martinez, 2002; Tavares, 2002; Roditakis *et al*., 2015).

The practice of using plant extracts is increasing in agriculture, especially in the organic product line and family farming for biological control and management systems. The effectiveness of the extracts is due to the presence of secondary metabolites, substances accumulated in small proportions in the plant tissues, which have several specific functions, in general biochemical defenses that act on insect behavior and physiology (Santos *et al*., 2017; Senthil-Nathan, 2013; Nishida, 2014). The 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

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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 culture, 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 establish these culture systems is the determination of procedures for the dedifferentiation of specialized plant cells into 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 aggregates of dedifferentiated cells, aiming at the determination 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).

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 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 (Santos and Souza, 2016).

This study describes the dedifferentiation of specialized internodal cells of *Capsicum annuum* cv. Yolo Wonder and evaluates the proliferation 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. Yolo Wonder 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 internodes were cut in explants of 1.0 cm length 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, 0.44, 2.22 and 11.10 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 percentage of callus induced per treatment (%CI), the percentage of the explant area covered by callus cells (ACCC), determined according to Mendonça *et al*.(2013), 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.

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RESULTS AND DISCUSSION

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. 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 the medium without growth regulators, callus induction was not observed, demonstrating the need of their supplementation for induction (Table 1). The positive interaction of 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 (100%), with the supplementation of 4.52 and 9.05 µM 2,4-D in combination with 2.22 µM BA and also of 9.05 and 18.10 µM 2,4-D with 0.44 µM BA. It can be inferred that, above 2.22 µM BA, there was saturation, or toxic effect of the growth regulator on the explants. 2,4-D alone, at 18.10 µM, also promoted 100% induction. In general an adequate balance between auxins and cytokinins is needed to the differentiation of specialized cells. The use of 2,4-D and BA has been reported 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 plus 2.22 µM BA. Khan *et al*. (2007) induced friable calluses in internodes of *C. annuum* cv. Pusa Jwala with 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. Kintzios *et al*. (1996) 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 same pattern observed for callus induction percentages was repeated for callus cell proliferation, evaluated by the explant area covered by callus cells (Table 2) and by the fresh mass of the explants (Table 3) after 42 days of *in vitro* cultivation. Evaluating simultaneously the three variables; %CI, ACCC and weight of the explants, the highest value, at a significant level, was observed with the combination of 4.52 µM 2,4-D + 2.22 µM BA, which resulted in 100% callus induction, 100% the explant area covered by callus cells, and calluses with an average weight of 2,374 mg.

Conclusion:

The culture medium supplemented with 4.52 µM 2,4-D and 2.22 µM BA resulted in the highest callus induction and proliferation. This combination of growth regulators induced callus in all the explants, which were totally covered by callus cells 42 days after inoculation, with the highest average weight of 2,374 mg per explant.

In vitro induction and proliferation of callus in internodes of *C. annuum* cv. Yolo Wonder can be successfully achieved in MS medium supplemented with 4.52 µM 2,4-D in combination with 2.22 µM BA.

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Table 1: Percentages of callus induction in internodal explants of *C. annuum* cv. Yolo Wonder in an MS medium supplemented with 2,4-D and BA, 42 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

*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 of the percentage of area covered by callus cells in internodal explants of *C. annuum* cv. Yolo Wonder in an MS medium supplemented with 2,4-D and BA, 42 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

*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 3: Average weight (mg) of internodal explants of *C. annuum* cv. Yolo Wonder in an MS medium supplemented with 2,4-D and BA, 42 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

*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.

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