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DEDIFFERENTIATION OF LEAF CELLS AND GROWTH PATTERN OF CALLUSES OF CAPSICUM FRUTESCENS CV. STROMBOLI

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ABSTRACT

In vitro cell suspension cultivation systems have been largely reported as safe and standardized methods of production of secondary metabolites with medicinal and agricultural interest. *Capsicum frutescens*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 objective of this study was to establish a protocol for dedifferentiation of leaf cells of the cultivar *C.frutescens* cv. Stromboli 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. 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 fresh 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 treatment that resulted in the highest %CI, ACCC and callus weight was 1.5 mg L^{-1} 2,4-D. The calluses produced were friable and whitish and their growth pattern followed a sigmoid shape. The deceleration phase started on the 39th day of cultivation.

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INTRODUCTION

Hot 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, 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 encourages the evaluation of their utilization as alternatives in the control of agricultural pests. The bioactivity of extracts and isolated substances from *Capsicum* species has been largely studied. 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); seed powder showed toxic effect against *Sitophilus zeamais* and *Callosobruchus maculatus*, insects that cause damage in stored maize and cowpea, respectively (Oni, 2011). Acaricidaleffect 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*, *Clavibactermichiganensis* 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). 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 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. frutescens* cv. Stromboli. 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 sub cultured 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 *Capsicumfrutescens*cv. Stromboli 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 35 days of cultivation, the plants were approximately 6 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; 1,0; 2,0; 3,0 and 4,0 mg L^{-1}) and 6-Benzylaminopurine(BA)(0; 1,0; 2,0; 3,0 and 4,0 mg L^{-1}) 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 (Kintzios et al., 2000; Kittipongpatana et al., 2007; Umamaheswari and Lalitha, 2007; Khan et al., 2011). 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 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. 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 regulator concentration that resulted in the highest callus cell proliferation; 1,5 mg L^{-1}2,4-D. The explants were incubated in a growth chamber under the mentioned conditions. In the subsequent 70 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 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 formation in the treatment where there was no supplementation with growth regulators; organogenesis (formation of buds) occurred in all treatments where there was supplementation of BA alone; and there was proliferation of callus cells in other treatments.

It was observed that 2,4-D alone or in combination with BA was satisfactory for the callus induction in leaf explants, resulting in 100% induction, in all combinations. Farias-Filho (2006) cites 2,4-D as essential for callus regeneration from pepper anthers, providing high callus production rates. According to Taiz and Zeiger (2013), the callus formation has been obtained considerably through the auxin 2,4-D alone, or in combination with the cytokinin BA. Forket et al. (2013) state that 2,4-D is a synthetic auxin that aids in the development of callus and cytokinins, with the aid of auxins, are propellants of cell division. Khan et al. (2011) when evaluating the influence of the combination of 2,4-D and BA on the callus induction in explants of *Capsicum annuum*cv PusaJwala., observed that the greatest proliferation of callus cells occurred in the presence of the two regulators of growth, obtaining better responses in the concentrations of 2.21 mg L^{-1} of 2,4-D + 0.4 mg L^{-1} of BA. Kittipongpatana et al. (2007) observed in leaf explants of *C. annuum* a positive effect of 2,4-D alone (1.0 mg L^{-1}) or in association with BA (1.0 mg L^{-1} 2, 4-D + 0.1 mg L^{-1} BA). Treatments supplemented with BA alone resulted in bud formation in 30% of explants. Otroshy et al. (2011) also observed the presence of buds when using 2.0 mg L^{-1} BA alone in nodal explants of *C. annuum*. Verma et al. (2013) aiming at the regeneration of *C. annuum* cv. California Wonder., obtained more efficient results in the combination of 6.0 mg L^{-1} BA + 0.3 mg L^{-1} IAA. In general, the presence of a cytokinin is essential for bud formation. Regarding the appearance of the calluses, the use of 2,4-D alone in all concentrations resulted in white and friable calluses.

However, when combined with BA, calluses presented a friable and compact aspect, and when BA was used alone, the proliferation of compact calluses occurred. Contrary results were obtained by Santos et al. (2014), who obtained white and friable calluses when using BA alone, at 1.0; 2.0 and 4.0 mg L^{-1}.Mangang (2014), when testing the effect of the combination of auxin and cytokinin on placental tissues of fruits of *C. chinense* cv. Umorok., obtained a better proliferation response of friable calluses with 2.0 mg L^{-1} 2.4-D + 0.5 mg L^{-1}KIN. Umamaheswari and Lalitha (2007) obtained the same response from leaf explants of *C. annuum* using these same concentrations. According to George et al. (2008), the texture and morphology of the callus, manipulated by variations in the constituents of the nutrient medium, produces soft, friable and moist calluses in a medium with a high concentration of auxin and low cytokinin, and if the relationship is reversed, it produces tissue calluses dry compact with small cells. According to Flores (2006), calluses with different growth rates and levels of differentiation (friable and compact) may differ in their ability to synthesize bioactive compounds. Friability is important for cell suspension cultures, as the cells separate when manipulated and divide easily in the culture medium, accelerating cell multiplication. For *C. frutescens* cv. Stromboli the treatments that were more efficient to obtain friable calluses were those supplemented with 2,4-D only.

Regarding the leaf area covered by callus cells (ACCC), the highest averages were observed in the treatment supplemented with 1.0 mg L^{-1} 2.4-D and in the treatment with 1.0 mg L^{-1} BA + 4.0 mg L^{-1} 2,4-D; in these treatments, all explants showed 100% of ACCC, resulting in a score of 4.00.

The areas covered by calluses were significantly larger when the explants were inoculated in the presence of 2,4-D, showing that the presence of this auxin is essential in the process of callogenesis. The same was observed by Guimarães (2015), working with *Piper permucronatum* leaf explants. As callus induction is dependent on a relatively balanced hormonal balance of auxins and cytokinins (Pinhal et al., 2011), in the case of Stromboli, probably, the supply of auxin at a concentration of 1.0 mg L^{-1} in the medium of culture was sufficient to balance the endogenous cytokinin content of the explant. It is worth mentioning that the addition of 2,4-D at a concentration four times greater (4.0 mg L^{-1}) was necessary to provide a similar response when in the presence of 1.0 mg L^{-1} of BA. Thus, the results indicate that the cytokinin (BA) used alone in the culture medium, together with the endogenous cytokinins of the leaf segment, possibly provided a high concentration of this regulator in relation to endogenous auxins, causing a decrease in callus formation. As for the fresh mass of the explants, the highest values were observed in treatments containing only 2,4-D. This auxin is frequently used to induce callogenesis and, in the case of Stromboli, leaf explants responded positively to its presence. The results of this work are similar to those obtained by Nogueira et al. (2008), Santos et al. (2005), Soares (2003), Bonilla (2002) and Conceição (2000) working with leaf segments of *Byrsonima intermedia*, *Salyxhumboldtiana*, *Inga vera*, *Rudgeaviburnoides* and *Derris urucu*, respectively. They observed that exogenous cytokinins are not always necessary and that many tissues develop in vitro only with a supply of auxins. Regarding the growth curve of the calluses, it was observed that the lag phase occurred until the 5th day, the exponential phase from the 6th to the 32nd day, the linear phase from the 33th to the 38th day, the deceleration phase from the 39th to the 64th day, and the decline phase from the 64th to the 70th day.

In the 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 (Santos et al., 2010).

Conclusion

Callus induction in leaf explants of *C. frutescens*cv. Strombolican be achieved in MS medium supplemented with 1,5 mg L^{-1} 2,4-D; callus cell from the 39th to the 64th day of cultivation are appropriate to start a cell suspension culture.

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