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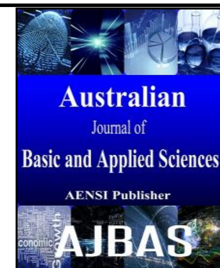

Growth Pattern of Friable Callus from *P. Carniconnectivum* Leaf Explants

Mauricio Reginaldo Alves dos Santos, Carolina Augusto de Souza, Milene de Castro Melo Guimarães, Caroline Vivian Smozinski, Glaura Mugrabe de Oliveira Magalhães, Wanessa de Oliveira Nogueira and Eloísa Santana Paz

Embrapa Rondonia, Box.127. Porto Velho. Brazil. 76815-800.

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ABSTRACT

Background: *Piper carniconnectivum* C. DC. (Piperaceae) is traditionally known as pimenta-longa (long pepper), native to the Brazilian Amazon. The secondary metabolites isolated from this species include flavonoids, caldensinic acid, E-phytyl hexadecanoate, β-sitosterol and stigmasterol mixture, phaeophytin, steroids, cyclopentenedione derivatives and the coumarin xanthyletin. Among its biological effects are the anti-inflammatory, antiplatelet, hepato- and neuroprotective actions, cytotoxicity to leukemia cells, besides its antifungal, antibacterial, leishmanicidal and herbicide activities. **Objective:** The objective of this study was the *in vitro* induction of friable callus in leaf explants of *P. carniconnectivum*, by determining the appropriate hormonal combination to produce high callus cell proliferation, and the identification of the growth pattern of the calluses focusing on the deceleration phase, when the callus cells must be subcultured into liquid medium in order to produce a cell suspension culture. **Results:** There was no interaction between the two growth regulators on callus induction or on LACC. From the regression analyses it was possible to infer the concentrations of 2,4-D (9.17 µM) and BA (10.37 µM) that resulted on the highest callus cell proliferation. The calluses produced were friable and whitish. The callus growth pattern followed a sigmoid shape. The deceleration phase started on the 40th day of culture. **Conclusion:** The proliferation of callus cells in leaf explants of *P. carniconnectivum* can be achieved in MS medium supplemented with 9.57 µM 2,4-D and 10.58 µM BA; callus cells at the 40th day of culture are appropriate to start a cell suspension culture.

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INTRODUCTION

The Piperaceae botanical family comprises of approximately 2,500 species and five genera, distributed in tropical and subtropical regions. In Brazil it is constituted of four genera and about 500 species. The genus *Piper* is the largest with more than 700 species, of which 170 are native to Brazil (Magevski *et al*., 2011). Several species of the *Piper* genus are native to the Amazon Rainforest and are notable producers of chemical compounds which have several biological effects on insects, fungi, bacteria, *Trypanossoma cruzi* (Navickiene *et al*., 2003; Dyer *et al*., 2004; Danelutte *et al*., 2005; Balbuena *et al*., 2009) and can also affect human health, as analgesic, anti-depressant, cytoprotective, anti-ulcer, anticonvulsant, anti-inflammatory, and antioxidant agents (Ahmad *et al*., 2010).

Piper carniconnectivum C. DC. (Piperaceae) is traditionally known as pimenta-longa (long pepper), native to the Brazilian Amazon (Alves *et al*., 2010). The major component of its leaf's essential oil is caryophyllene oxide (21.3%); the major components in the stem essential oil are spathulenol (23.7%) and α-pinene (19.0%) (Facundo *et al*., 2006). From its aerial parts were isolated three flavonoids (Alves *et al*., 2008), caldensinic acid, E-phytyl hexadecanoate, β-sitosterol and stigmasterol mixture, phaeophytin (Alves *et al*., 2010), and two steroids (Facundo and Braz-Filho, 2004). From its roots were isolated four flavonoids, three natural cyclopentenedione derivatives and the coumarin xanthyletin (Facundo *et al*., 2004), later reported for its biological potential, including antiplatelet, antifungal and herbicide activities (Hayasida *et al*., 2011). A cyclopentenedione derivative was synthetized in order to access additional derivatives with potential relevance to biological studies (Dias *et al*., 2005) and later tested *in vitro* against *Leishmania amazonensis*, being considered as a promising molecule for the development of leishmanicidal drugs (Paes-Gonçalves *et al*., 2012). Cyclopentenediones are known to have antibacterial and antifungal effects, besides anti-inflammatory, hepato- and

Corresponding Author: Mauricio Reginaldo Alves dos Santos, Embrapa Rondonia, Box. 127. Porto Velho. Brazil. 55-69-39012525.
E-mail: mauricio.santos@embrapa.br

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neuroprotective effects (Sevcíková *et al*., 2014). Flavones isolated from leaves showed cytotoxic activity on seven out of twelve leukemia cell lines (Freitas *et al*., 2014).

Secondary metabolites can be efficiently produced *in vitro*. Research to date has succeeded in producing a wide range of valuable secondary phytochemicals in unorganized callus or suspension cultures (Hussain *et al*., 2012). The major advantages of a cell culture system over the conventional cultivation of whole plants are: (1) Useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions; (2) Cultured cells would be free of microbes and insects; (3) The cells of any plants could easily be multiplied to yield their specific metabolites; (4) Automated control of cell growth and rational regulation of metabolite processes would reduce the labor costs and improve productivity; (5) Organic substances are extractable from callus cultures (Vanisree *et al*., 2004).

The objective of this study was the *in vitro* induction of friable callus in leaf explants of *P. carniconnectivum*, by determining the appropriate hormonal combination to produce high callus cell proliferation, and the identification of the growth pattern of the calluses focusing on the deceleration phase, when the callus cells must be subcultured into liquid medium in order to produce a cell suspension culture. The calluses thereby produced will be used for the further establishment of cell suspension cultures of *P. carniconnectivum* and to assess the feasibility of *in vitro* production of some important secondary metabolites.

MATERIALS AND METHODS

Plant materials and sterilization:

Young leaves were excised from flowering, healthy, and disease free *P. carniconnectivum* stock plants from Embrapa (Brazilian Agricultural Research Corporation), in Porto Velho, RO, Brazil. The plants were six months old and approximately 90 cm tall, maintained in a shaded greenhouse under a maximum photosynthetic photon flux density of 300 $\mu mol.m^{-2}.s^{-1}$ and average temperature of 25.6°C. After washing with running tap water and a detergent agent for five minutes, the leaves were surface-sterilized in 70% (v/v) ethanol for 1 minute and soaked in a 1.0% (v/v) sodium hypochlorite solution for 1 minute, and then rinsed three times with sterile water. Explants were produced by cutting the leaves into 1 cm^2 pieces in sterile petri dishes.

Callus induction, proliferation and growth:

Leaf explants were individually transferred with the adaxial surface up, to test tubes (25 mm x 150 mm) containing 10 mL of an MS (Murashige & Skoog, 1962) basal culture medium supplemented with 3% (w/v) sucrose, 0.6% (w/v) agar and factorial combinations of 2,4-Dichlorophenoxyacetic acid (2,4-D) (0, 4.52, 9.05, or 18.10 µM) and 6-Benzylaminopurine (BA) (0, 4.44, 8.88, or 17.76 µM), totaling 16 treatments. The pH of the medium was adjusted to 5.8 before the addition of agar followed by autoclaving at 121°C for 20 minutes. Callus formation was evaluated weekly until the 49th day, by assessing the number of callus induced per treatment and the leaf area covered by callus cells (LACC), according to Mendonça *et al.* (2013). Subsequently, new explants were inoculated at the determined hormonal combination in order to induce high callus cell proliferation and, every seven days during a period of 63 days samples were weighed in order to determine the callus growth pattern.

Experimental design and evaluation:

All the cultures were incubated in a growth chamber at 25±2°C under light provided by cool white fluorescent tubes (50 $\mu mol.m^{-2}.s^{-1}$). Treatments were arranged in a completely randomized design using four replicates of three explants for callus induction and LACC, and three replicates for determination of the callus growth curve. Variance analyses and polynomial regression were performed by using the Assistat 7.7 statistical program. Maximum values of the regression curves and corresponding concentrations of growth regulators were estimated according to Purcell and Varberg (1984).

RESULTS

There was no callus induction on the medium without growth regulators. All the treatments where both regulators were supplemented resulted in callus induction. Interestingly, according to the variance analyses, the effects of 2,4-D and BA were individually significant on callus induction, yet there was no significant interaction between the two regulators (Table 1).

Considering the lack of interaction between the effects of both regulators, they were evaluated separately by polynomial regression. Callus induction by 2,4-D and BA were represented by quadratic regressions (Figures 1 and 2). The estimated maximum percentage of callus induction by 2,4-D was 77.62% and the corresponding 2,4-D concentration was 8.77 µM. In relation to the effect of BA the estimated maximum percentage of induction was 73.51% and the corresponding BA concentration was 10.16 µM.

Regarding LACC, the effects of the two growth regulators also did not interact significantly (Table 2) and likewise could be represented by quadratic equations (Figures 3 and 4). The estimated maximum percentages of LACC were 48.59 and 47.79%, corresponding respectively to the supplementation of the media with 9.57 µM 2,4-D and 10.58 µM BA.

The growth pattern of the calluses followed a sigmoid shape (Figure 5). It was possible to identify a lag phase from the day of inoculation until the 13th day of culture, an exponential phase from the 13th to the 35th day, a linear phase from the 35th to the 40th day, a deceleration phase from the 40th to the 52nd

day, a stationary phase from the 52nd to the 56th day, and a decline phase from the 56th to the 63rd day.

DISCUSSION

Callus induction and proliferation:

In the present study there was callus induction without interaction of the regulators and even where only 2,4-D or BA was present. However, in spite of the lack of interaction, the percentage of induction was higher where both regulators were supplemented, indicating a synergistic effect. The same occurred to the LACC, where the regulators had no interaction. To date, the approaches regarding callus induction and callus cell proliferation on *Piper* species present interaction among the growth regulators supplemented to the media. In general, callus induction is supposed to be reached with a hormonal balance guaranteed by combinations of exogenous auxins, cytokinins or gibberellins.

The effective combinations of regulators for callogenesis are quite variable among *Piper* species. In the present research the highest callus induction in leaf explants of *P. carniconnectivum* should be achieved with a combination of 8.77 µM 2,4-D and 10.16 µM BA, whose outcome were 77.62% and 73.51% of the explants with callus induction, respectively. Much higher concentrations of auxins were used by Santiago (2003), who observed the induction of friable green calluses in 98.3% of the leaf explants of *P. hispidinervium* submitted to a medium with 27.14 µM 2,4-D, 8.88 µM BA and 2.69 µM NAA. Studying the same species, Valle (2003) found different results, with a pro-cytokinin hormonal balance, reaching the highest proliferation of callus cells with the combination of 22.62 µM 2,4-D and 45.25 µM BA or 26.85 µM NAA and 45.29 µM BA. Distinctly lower cytokinins concentrations were effectively used by Ahmad *et al.* (2010), who found 93 and 90% of callogenesis, respectively, in explants of *P. nigrum* cultured on MS medium supplemented with either 2.22 µM BA or 4.44 µM BA in combination with 5.37 µM NAA. Kelkar *et al.* (1996) achieved 100% of callus induction in leaf explants of *P. colubrinum* by supplementing the media with 2.26 µM 2,4-D plus 4.44 or 8.88 µM BA, 2.69 µM NAA plus 4.44 or 8.88 BA, and 5.37 µM NAA plus 2.22 µM BA.

In the present study the highest callus cell proliferation should be reached with a combination of 9.57 µM 2,4-D and 10.58 µM BA, as it can be inferred from the %LACC observed in the isolated effect of both regulators, 48.59% and 47.79%, respectively. Delgado-Paredes *et al.* (2013) identified 50 to 67% of LACC in *P. crassinervium* by using 1.07 µM or 10.74 µM NAA and 100% of LACC in *P. regnellii* by using 0.05 µM NAA in addition to 4.44 µM BA. Balbuena *et al.* (2009) achieved the highest callus cell proliferation in *P. solmsianum* leaf explants with 0.90 µM 2,4-D in combination with 8.88 µM BA. Dominguez *et al.* (2006) observed the induction of fast growing, friable, yellow-green calluses in *P. auritum* with 9.05 µM 2,4-D plus 6.97 µM Kin. Danelutte *et al.* (2005) observed the highest callus induction in *P. cernuum* leaf explants with 0.90 µM 2,4-D plus 1.44 µM GA_3 and in *P. crassinervium* with 5.37 µM NAA plus 0.88 µM BA.

All these diverse responses found among *Piper* species in relation to the adequate hormonal balance needed for callus induction reflect the genotypic diversity of the genus, and its effects on the physiological behavior of each species. As was mentioned by George *et al* (2008), due to genotypic specificity, media and cultural environment often need to be varied from one genus or species of plant to another and even closely related varieties of plants can differ in their cultural requirements.

Callus growth:

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: 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 the current approach, the evaluation of the callus growth curve followed a typical sigmoid shape with the deceleration phase starting on the 40th day of culture. This is the moment when callus cells should be transferred to a liquid medium in order to establish cell suspension cultures. Similarly, 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. Differently, Balbuena *et al.* (2009) used callus cells of *P. solmsianum* on the 24th day of culture to initiate cell suspension cultures.

CONCLUSIONS

The proliferation of callus cells in leaf explants of *P. carniconnectivum* can be achieved in MS medium supplemented with 9.57 µM 2,4-D and 10.58 µM BA; callus cells at the 40th day of culture are appropriate to start a cell suspension culture.

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Table 1: Variance analyses of the effect of 2,4-D and BA on callus induction in leaf explants of *P. carniconnectivum*, 49 days after inoculation.

Source	Degrees of Freedom	Mean Square	F
2,4-D	3	9.521	**
BA	3	6.188	**
Interaction (2,4-D x BA)	9	1.257	ns

** - significant at 1% probability by F test; ns - not significant.

Table 2: Variance analyses of the effect of 2,4-D and BA on *P. carniconnectivum* leaf area covered by callus cells (LACC) , 49 days after inoculation.

Source	Degrees of Freedom	Mean Square	F
2,4-D	3	5.597	**
BA	3	7.261	**
Interaction (2,4-D x BA)	9	0.906	ns

** - significant at 1% probability by F test; ns - not significant.

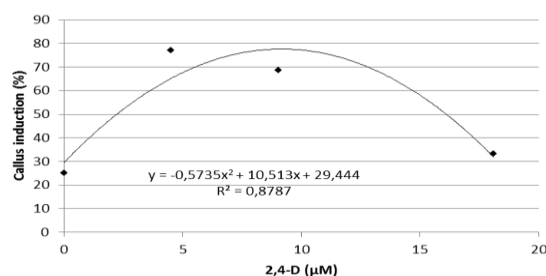

Fig. 1: Percentage of callus induction in leaf explants of *P. carniconnectivum* in MS medium supplemented with 2,4-D, 49 days after inoculation.

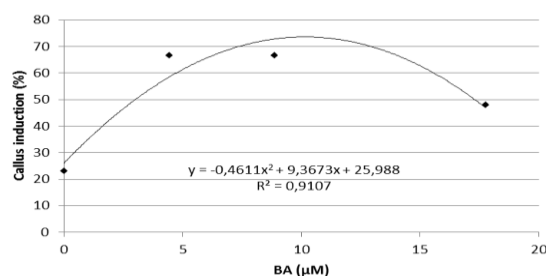

Fig. 2: Percentage of callus induction in leaf explants of *P. carniconnectivum* in MS medium supplemented with BA, 49 days after inoculation.

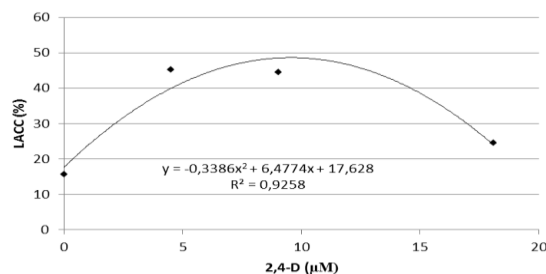

Fig. 3: Leaf area covered by callus cells (LACC) in explants of *P. carniconnectivum* in MS medium supplemented with 2,4-D, 49 days after inoculation.

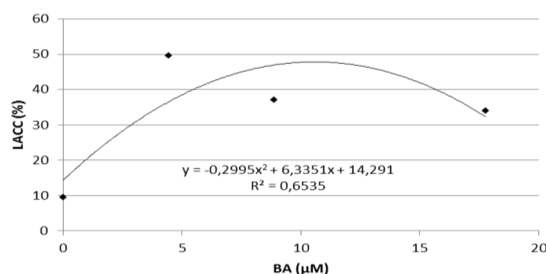

Fig. 4: Leaf area covered by callus cells (LACC) in explants of *P. carniconnectivum* in MS medium supplemented with BA, 49 days after inoculation.

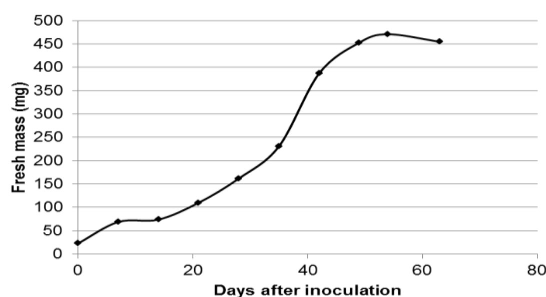

Fig. 5: Growth curve of *P. carniconnectivum* leaf calluses in MS medium supplemented with a combination of 9.17 µM 2,4-D and 10.37 µM BA.

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