Establishment and content of sugars and phenols in *Physalis* callus obtained from different explants and concentrations of bap and naa

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ABSTRACT. Obtaining cells of *Physalis pubescens* is of interest for studies of primary and secondary metabolic pathways, in the search for new active molecules. Our objectives were to evaluate the regeneration potential of explants from different parts of the plant, growth regulators to be used, and the determination of the growth curve of the callus. We used explants of leaf, root, stem and petiole, cultured on Murashige and Skoog medium with different concentrations of 6-benzylaminopurine and α-naphthaleneacetic acid. The explants from stem and petiole had a higher regeneration potential of the shoot to the treatment with 0.5 mg L^{-1} 6-benzylaminopurine, and the explants of leave and root emitted more roots, with lower production of callus. The tests showed that the regeneration of the whole plant should be done in two steps: cultivation for shoot regeneration and transplantation to a new rooting medium. The growth of callus showed five distinct phases, with accumulation of phenols in the final stages of growth. The levels of soluble sugars increased with age, while reducing sugars showed variations, with higher concentrations in the initial stages of cultivation, with fall and rise again at the final evaluation (28th day).

Keywords: cell, growth, phenols, sugars.

Estabelecimento e conteúdo de açúcares e fenóis em calos de *Physalis* obtidos de diferentes explantes e concentrações de bap e ana

RESUMO. A obtenção de células de *Physalis pubescens* é de interesse para estudos de vias metabólicas primárias e secundárias na busca de novas moléculas ativas. Os objetivos deste trabalho foram avaliar o potencial de regeneração de explantes de diferentes partes da planta, os reguladores de crescimento a serem utilizados e a determinação da curva de crescimento dos calos. Foram utilizados explantes de folhas, raízes, caules e pecíolos, cultivados em meio Murashige e Skoog em diferentes concentrações de 6-benzilaminopurina e α-ácido naftaleno acético. Os explantes provenientes de pecíolos e caules apresentaram maior potencial de regeneração da parte aérea com o tratamento com 0,5 mg L^{-1} de 6-benzilaminopurina, sendo que os explantes de folhas e raízes emitiram mais raízes com menor produção de calos. Os ensaios demonstraram que a regeneração da planta inteira deve ser feita em duas etapas: cultivo para regeneração da parte aérea e repicagem para novo meio de enraizamento. A curva de crescimento de calos apresentou cinco fases distintas, com acúmulo de fenóis nos estágios finais de crescimento. Os teores de açúcares solúveis totais foram crescentes com a idade, enquanto açúcares redutores apresentaram variações com maiores concentrações nas etapas iniciais do cultivo, com queda e novo aumento no final da avaliação.

Palavras-chave: células, crescimento, fenóis, açúcares.

Introduction

The genus *Physalis* includes about 90 species widely distributed in tropical and subtropical regions of the world (DAMU et al., 2007). Many of them are cultivated aiming fruit production, but other parts of the plant are used in folk medicine, in the treatment of several illnesses such as malaria, asthma, hepatitis, rheumatism, dermatitis, etc. (DI STASI et al., 1989).

Physalis pubescens L. is a subshrub with 30 cm height, distributed throughout the Americas and Old World tropics (RUFATO et al., 2008). It can be propagated by seed, cutting or micropropagation, this last being more rapid to obtain high quality seedlings.

The culture of plant tissues can begin from any part of the plant and the choice of the type of explant will depend on the expected results, availability of the plant material, and responsiveness of the material to the culture medium, growth regulators and their concentrations, which can be used alone or combined, considering the desired product (CARVALHO, 2006).

The cell culture, as callus or cell suspension, is an important tool that allows studying several aspects of physiology, metabolism and plant cell development, in a simplified form with the possibility of controlling several factors.

Cell growth can be evaluated in two ways: at individual (cell cycle) or population level (growth cycle). For the determination of this latter, the most common technique is the determination of the cell mass. According to Soares et al. (2003), in order to establish the growth curve of the callus of a given species is important to identify the phases when occur processes essential to the kinetics of growth, by setting the exact time for transfer to a new medium.

Another important issue is the knowledge of biochemical changes occurring during the cell development that may aid in the morphogenetic process during the cultivation, besides allowing the identification of substances of the primary and secondary metabolism.

Primary metabolism products are essential to plants and carbohydrates stand out due to their functions as energy source (SANTOS et al., 2010) while secondary metabolites like phenols and indicate different responses to environmental conditions (CASTRO et al., 2008).

The present study aimed to evaluate the potential for forming callus from different parts of the plant (root, leaf, stem, petiole) of *P. pubescens*, under different concentrations of growth regulators (BAP and NAA), evaluating other results, such as the formation of shot, and roots, and/or regeneration of the whole plant, as well as to determine the cell growth curve and the formation of total phenols, total soluble sugars, and reducing sugars at each phase of cell growth.

Material and methods

Seeds of *P. pubescens* were placed on vials containing MS culture medium (MURASHIGE; SKOOG, 1962) to obtain explants. Initially, seeds underwent disinfection with 70% ethanol (v/v) for 2 minutes and sodium hypochlorite at 0.2% active chlorine (v/v) for 20 minutes. The vials were incubated at room temperature between 26 ± 2°C and photoperiod of 12 hours (light intensity of 240 lux).

Plants obtained 60 days after sowing have provided explants of leaf, petiole, stem and roots, which were cultivated in MS medium with different concentrations of growth regulators (NAA – α-naphthaleneacetic acid and BAP – 6-benzylaminopurine), forming the following

treatments: 1 (control, without growth regulator), 2 (0.5 mg L^{-1} NAA), 3 (0.5 mg L^{-1} NAA + 0.25 mg L^{-1} BAP), 4 (0.5 mg L^{-1} NAA + 0.5 mg L^{-1} BAP), 5 (0.5 mg L^{-1} BAP) and 6 (0.25 mg L^{-1} NAA + 0.5 mg L^{-1} BAP). Each treatment consisted of 5 vials (replications) being considered each vial a replication with four explants each. The vials were kept under the same conditions of temperature and light above mentioned. It was used a completely randomized experimental design, and the results obtained were subjected to Tukey's test at 5%, without prior transformation of the data. The evaluations were performed every 7 days (7, 14, 21, 28 and 35 days), by counting the explants that formed leaves and/or roots or callus.

Callus were removed from vials and weighed from the 7th days after inoculation to the 28th day, at 7 days-intervals (4 evaluations).

To obtain cell mass for biochemical analyses and determination of growth curve (fresh weight), it was selected the best treatment for forming callus, the treatment 6 (0.25 mg L^{-1} NAA and 0.5 mg L^{-1} BAP), grown under the same conditions.

Callus after mass determination were submitted to biochemical analyses to quantify total sugars (TS) by the phenol-sulfuric acid method (DUBOIS et al., 1956) and reducing sugars (RS) as described by Miller (1959), both with glucose as standard, and to determine total phenols by the method adapted from Bieleski and Turner (1966) and Jennings (1981).

Results and discussion

In the trials conducted, *P. pubescens* had characteristics of indirect organogenesis. In the assessments of the several combinations of BAP and NAA, the treatment 5 (0.5 mg L^{-1} BAP) presented the best combination to induce shooting with the use of petiole explants (90% of shoots) and stem explants (nodal segments with 70%) (Table 1), while other plant materials and treatments were little or no effective to produce buds.

When callus production was evaluated, the same explants (stems and petioles) showed great capacity to form callus in different treatments (Table 2). The better combinations of plant regulators were treatments 4 and 6, but 2 and 3 resulted in not significantly different values. Although different concentrations were used, in others works with palm grass [*Opuntia ficus-indica* (L.)], authors have obtained similar results with MS culture medium with 1 mg L^{-1} BAP (FROTA et al., 2004), and in *Syngonanthus mucugensis* Giulietti where authors suggested that nodal segments were responsive to formation of friable callus, with significant production with 1.78 and 3.55 μM BAP concentrations (SANTOS et al., 2008).

Table 1. Mean values (%) of regeneration of shoot of *Physalis pubescens* using different explants, combinations and concentrations of BAP and NAA.

Explants		Treatments					
		1	2	3	4	5	6
Formation of bud	Stem	10 aB	10 aB	5 aB	15 aB	70 bA	10 aB
	Petiole	15 aB	10 aB	5 aB	0 aB	90 aA	5 aB
	Leaf	0 aA	0 aA	0 aA	0 aA	0 cA	0 aA
	Root	0 aA	0 aA	0 aA	0 aA	0 cA	0 aA

Mean values followed by the same letter are not significantly different. Uppercases in the rows and lower cases in the columns. Tukey's test at 5% probability.

Table 2. Mean values (%) of formation of callus of *P Physalis pubescens* using different explants, combinations and concentrations of BAP and NAA.

Explants		Treatments					
		1	2	3	4	5	6
Formation of callus	Stem	0 aC	60 aB	75 aAB	90 aA	15 aC	100 aA
	Petiole	0 aB	80 aA	85 aA	100 aA	0 aB	100 aA
	Leaf	0 aA	15 bA	15 bA	5 bA	0 aA	10 bA
	Root	10 aA	5 bA	5 bA	5 bA	5 aA	25 aB

Mean values followed by the same letter are not significantly different. Uppercases in the rows and lower cases in the columns. Tukey's test at 5% probability.

Al Khateeb et al. (2012) verified that budding were induced in callus of *Cichorium pumilum* Jacq. when the MS medium was supplemented with 1.5 mg L^{-1} BAP or kinetin. Other results are in accordance with the auxin: cytokinin ratio in the formation of shoot or roots, as observed by Ghimire et al. (2012) in *Solanum aculeatissimum* Jacq. with medium supplemented with 0.1 mg L^{-1} NAA and 2.0 mg L^{-1} BAP. Likewise, these authors showed that petiole explants responded better to the induction, especially with NAA (0.1 mg L^{-1}) and 1.0 mg L^{-1} thiadiazuron. Combinations with higher concentration of cytokinin tend to promote the development of the shoot, as also observed by Dode et al. (2003) with concentrations with BAP and NAA 5.0 and 0.2 mg L^{-1}, respectively, being the lowest concentration of BAP (1.0 mg L^{-1}) also effective. In *Melissa officinalis* L. buddings were also better induced with 2.0 mg L^{-1} BAP (MOHEBALIPOUR et al., 2012), as well as in *Dioscorea multiflora* Grised (0.1 mg L^{-1} BAP) (SOUZA et al., 2011).

Regarding the formation of roots, significant difference was found between treatments and when different explants were used. Leaf explants were not adequate to produce roots without plant regulators (treatment 1) and in the treatment 5 (Table 3). Depending on the type of explant, the results suggested an endogenous balance between auxin: cytokinin promoting the rhizogenesis, which was observed in the control (treatment 1), where explants, except for leaf ones, were able to give rise to roots after a low cell growth (callus).

According to Al Khateeb et al. (2012) high concentrations of indole butyric acid (IBA) or NAA inhibit the formation of roots in callus obtained from leaf explants of *C. pumilum* Jacq. In the case of seeds of marigold, the rooting occurred in the absence of BAP, without the need to add NAA, and under its presence there was a decrease in the process (BEVILACQUA et al., 2011). In *D. multiflora*, authors did not register satisfactory results in inducing the rooting, using NAA or IBA in several concentrations (SOUZA et al., 2011). Inhibition of the formation of roots was also observed in the presence of NAA combined with different concentrations of BAP (1 to 5 mg L^{-1}) in the in vitro propagation of *O. basilicum* (DODE et al., 2003). Although the difficulty for rooting is a problem usually found in woody species, herbaceous may also present unsatisfactory results, as observed in the present study and pointed out by other authors (SOUZA et al., 2011; SOUZA; PEREIRA, 2007), not only due to the endogenous levels of phytohormones but the addition of growth regulators at inadequate proportions and/concentrations.

The Table 1 shows that the regeneration of *P. pubescens*, with BAP and NAA under tested concentrations, should be done in two steps, i.e., first the cultivation to obtain the shoot, when only BAP is enough to induce shooting, and then the transplantation to a rooting medium, when the purpose is the plant regeneration. Probably other auxins have better results in inducing rooting in *Physalis* than does the NAA.

Stem and petiole explants presented a better potential to form callus but petiole was better plant material in more than one treatment, as can be observed in the Table 2. Leaf and root explants were not the most indicated to obtain shoot, but presented good results in obtaining roots (treatment 3 for leaf, and 4 and 6 for root explants), being superior to petiole and stem. For the main goal of this study, i.e., obtaining callus, both material were quite below the other two (petiole and stem) in almost all treatments (Table 2).

Table 3. Mean values (%) of regeneration of roots of *Physalis pubescens* using different explants, combinations and concentrations of BAP and NAA.

	Explants	Treatments					
		1	2	3	4	5	6
Formation of root	Stem	30 aA	15 aA	15 aA	25 abA	10 aA	10 bA
	Petiole	30 aA	15 aA	25 aA	35 abA	5 aA	30 abA
	Leaf	0 bB	30 aAB	45 aA	10 bAB	0 aB	40 abAB
	Root	30 aA	20 aA	25 aA	55 aA	35 aA	55 aA

Mean values followed by the same letter are not significantly different. Uppercases in the rows and lower cases in the columns. Tukey's test at 5% probability.

Owing the great diversity of natural compounds, the cell culture is an important tool for studies involving processes of formation of these products, as well as the use of elicitors of their synthesis. The formation of callus or the differentiation of plant tissues depends on the auxin: cytokinin ratio in the culture medium, while a high ratio stimulates the rooting, a low ratio leads to the formation of shoot. At intermediate levels of both plant regulators, cells divide and form callus, i.e., undifferentiated cells (TAIZ; ZEIGER, 2010).

In order to assess the cell growth, it was used the best result obtained in the experiment of cultivation with different levels of NAA and BAP, i.e., the treatment 6, with stem sections of *P. pubescens*. Callus were weighed in periods of 7 days, being obtained a cell growth curve that follow the same pattern described for several species (SANTOS et al., 2010; SOOMRO; MEMON, 2007; STELLA; BRAGA, 2002) of three distinct phases: lag, exponential and stationary (Figure 1). The lag phase in which the explant cells prepare for cell division, is characterized by the mobilization and synthesis of metabolites for the process, occurred until the 7th day. The exponential phase, with the maximum cell division, was observed between the 7th and 21st day.

According to Lima et al. (2007), the phase lag can be considered as energy producer, and the exponential phase as biosynthetic, i.e., producer of complex chemical compounds (protein and nucleic acid).
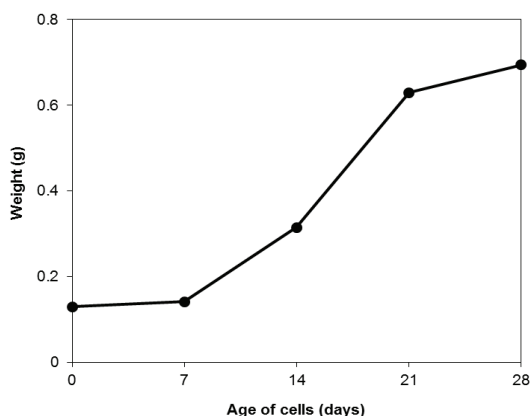

Figure 1. Growth curve of cells of *P. pubescens* cultivated in MS medium added with 0.25 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. Mean value of 4 repetitions.

Within the growth phase, there was a slight deceleration of growth, called by some authors as linear phase, when cells decrease the division process and start the volume increase (CASTRO et. al., 2008). In the present study the reduction in division may have occurred between the 14th and 21st day of cultivation.

From the 21st inoculation day, cell growth was not significant, being considered as the beginning of the stationary phase, when decrease both the division and increase in cell volume and start the synthesis and accumulation of compounds of the secondary metabolism (BANTHORPE, 1994). For maintaining the cultivations of *P. pubescens*, callus should be transplanted before the 21st day, because they are in the process of cell division and possibly do not accumulate substances that may be toxic or inhibit growth (SMITH, 1992; BANTHORPE, 1994). Between the 21 and 28 days, cells have presented a color change, becoming slightly darker, indicating cell death and probable accumulation of compounds of the secondary metabolism.

As a function of the possible accumulation of secondary compounds, were determined the content of total phenols, but it was only possible from the 14th day due to the low cell mass hitherto. These compounds have accumulated with advancing age of the cells (28 days; Figure 2). Castro et al. (2009) also observed higher concentration of total phenols in callus of *Stryphnodendron adstringens* (Mart.) Coville over time, and suggested a mechanism of degradation of these compounds in the beginning of callogenesis, which according to Yazaki et al. (1991) favor new cell divisions, preventing thus the oxidation of the callus.

In relation to total phenols, the antagonistic relationship between primary and secondary metabolism in cell cultures was shown, where the synthesis and accumulation of secondary compounds occur by the end of the cell growth (BANTHORPE, 1994).

The content of total soluble sugars (TS) increased with the age of cells, with the highest concentration on the 28th days (Figure 3), similar

to observed for callus of other plant species, such as coffee tree (*Coffea arabica* L., cultivar Rubi) (SANTOS et al., 2003), lychee tree (*Litchi chinensis* Sonn.) (MESQUITA et al., 2002) and *Jatropha curcas* L. (SANTOS et al., 2010).
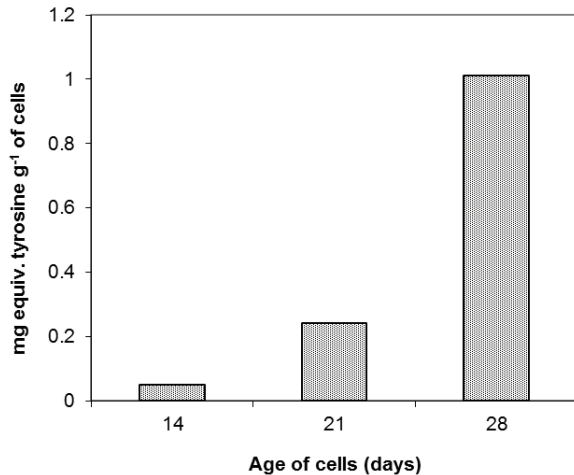

Figure 2. Total phenols in cells of *P. pubescens* cultivated in MS medium added with 0.25 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. Mean value of 4 repetitions.
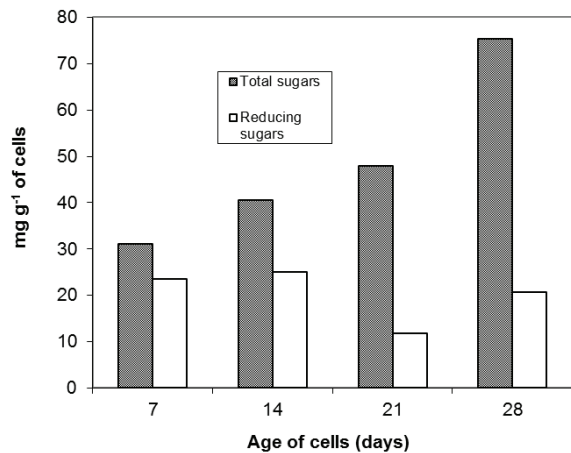

Figure 3. Total sugars (TS) and reducing sugars (RS) of cells (callus) of *P. pubescens* cultivated in MS medium added with 0.25 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. Mean value of 4 repetitions.

As for reducing sugars (RS), higher concentrations were observed in the initial stages (7 and 14 days old) (Figure 3), periods corresponding to the exponential phase of cell growth. Despite the different periods of evaluation and species, Santos et al. (2003) with coffee, and Santos et al. (2010) with callus of *J. curcas*, have observed similar behaviors, with variations in the levels of reducing sugars with advancing age, being greater in the early stages of cultivation, with later reductions and increases. According to Nogueira et al. (2006) higher values of RS in the beginning of cultivations can be

associated with explants, i.e., their reserves. Afterwards, the absorption of sucrose from the culture medium and breakdown into monosaccharides could explain the increase after exhausting initial reserves.

Conclusion

Petiole and stem explants have proven to be the most suitable for forming buds and callus. For the regeneration of the whole plant, the cultivation should be done in two steps with the first aiming the formation of shoot and the second for inducing rooting, probably with other auxin, since the NAA was not effective for this or even combined with other cytokinins.

The content of total soluble sugar has increased during the development of calluses, indicating the absorption from the medium and synthesis of new carbohydrates resulting in growth with variation in the concentration of reducing sugars. Some sugars can be derived from the reserve existing in the explants, at the beginning of the cultivation and later metabolism of carbohydrate obtained in the cultivation medium, varying according to the use in other cell processes.

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